Universidade de Aveiro Departamento de Química
Ano 2019

Fernanda do Carmo Machado Silva

Efeito da alta-pressão na qualidade e estabilidade de emulsões alimentares

High pressure effect on the quality and stability of food-grade emulsions

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho a minha mãe por todo o apoio, força e orientação ao longo de todos estes anos.

o júri / the jury

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Palavras-chave Natas, alta pressão, emulsão, pasteurização térmica, ultrassom

Resumo

A primeira parte deste trabalho, realizada em Portugal, teve como objetivo avaliar os efeitos de uma tecnologia não térmica, designada alta pressão (HPP), como possível alternativa ao tratamento térmico convencional, no aumento do prazo de validade de natas, um produto altamente perecível. A nata foi submetida à alta pressão (450 MPa por 5 min e 600 MPa por 5 e 15 min) e à pasteurização térmica, e armazenada durante 52 dias sob refrigeração (4 °C), para comparar os efeitos de ambos os tratamentos na sua carga microbiana (microrganismos endógenos e inoculados) e parâmetros físico-químicos. Diferentemente das amostras de HPP a 600 MPa, no 51º dia de armazenamento, os microrganismos endógenos das amostras termicamente pasteurizadas já tinham ultrapassado 6.00 log CFU/mL. Além disso, a alta pressão também foi capaz de reduzir (p<0.05) *Escherichia coli* e *Listeria innocua* inoculadas a contagens abaixo do limite de deteção (1.00 log CFU/mL). Em geral, pH, cor, viscosidade e ácidos gordos não foram significativamente alterados (p>0.05) pelas diferentes condições de processamento, exceto os compostos voláteis, que apresentaram uma tendência para aumentar ao longo do período de armazenamento. Estes resultados sugerem o HPP como um potencial substituto à pasteurização térmica convencional, podendo resultar num aumento de prazo de validade da nata.

A segunda parte desta tese, realizada em Itália, teve como objetivo avaliar a possibilidade de utilizar a tecnologia de ultrassons para obter uma emulsão estável, com altos valores nutricionais e microbiologicamente segura por HPP, obtida a partir de azeite extra-virgem (EVOO) (o tipo mais valioso de azeite) de diferentes teores de polifenóis, usando reduzidas quantidades de emulsificante. Observou-se que para obter uma emulsão estável por ultrassons foi necessário utilizar um emulsificante, neste caso mono- e diglicerídeos de ácidos gordos. Além disso, EVOOs com alto teor de polifenóis produziram emulsões mais estáveis. No entanto, o conteúdo em polifenóis não pareceu ter um efeito sobre as propriedades reológicas da emulsão. Após otimização das condições de ultrassons e as percentagens de cada ingrediente usado para obter a emulsão, foi possível obter uma emulsão estável. Em termos de estabilidade microbiológica, o HPP (500 MPa por 5 min) foi capaz de inibir ou retardar o crescimento microbiano ao longo do tempo (20 dias), mantendo as emulsões estáveis mesmo após armazenamento à temperatura ambiente.

Keywords Dairy cream, high pressure, emulsion, thermal pasteurization, ultrasound

Abstract

The first part of this work, carried out in Portugal, aimed to evaluate the effects of a non-thermal technology, known as high pressure processing (HPP), as a possible alternative to the conventional heat treatment, in extending dairy cream's, a highly perishable dairy product, shelf-life. Cream was subjected to HPP (450 MPa for 5 min and 600 MPa for 5 and 15 min) and to thermal pasteurization and then stored for 52 days under refrigeration (4 °C), in order to compare the effects of both treatments on cream's microbiology (endogenous and inoculated microorganisms) and physicochemical parameters. Unlike HPP samples at 600 MPa, by the 51st day of storage, endogenous microorganisms of thermally pasteurized samples had already surpassed 6.00 log CFU/mL. Furthermore, HPP was also able to reduce (p<0.05) inoculated *Escherichia coli* and *Listeria innocua* to counts below the detection limit (1.00 log CFU/mL). In general, pH, colour, viscosity and fatty acids were not significantly altered (p>0.05) by the different processing conditions, with the exception of volatile compounds, which presented a tendency to increase throughout storage period. These results hint HPP as a potential replacement of conventional thermal pasteurization regarding cream's shelf-life extension.

The second part of this thesis, carried out in Italy, aimed to evaluate the possibility of using ultrasounds technology to obtain a stable emulsion, with high nutritional values and microbiologically safe by HPP, based on extra-virgin olive oil (EVOO) (one of the most valuable type of olive oil) with different polyphenol content, using reduced amounts of emulsifier. It was observed that to obtain a stable emulsion by ultrasounds it was necessary to use an emulsifier, in this case mono- and diglycerides of fatty acids. Furthermore, EVOOs with high polyphenols content yield more stable emulsions. Nevertheless, the polyphenols content did not seem to have an effect on emulsion's rheological properties. After optimizing the ultrasounds conditions and the percentages of each ingredient used to make the emulsion, it was possible to obtain a stable emulsion. Regarding microbial stability, HPP (500 MPa for 5 min) was able to inhibit or slow microbial growth over time, keeping the emulsions stable even after storage (20 days) at room temperature.

CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Designation
a_w	Water activity
α-La	α-lactalbumin
β-Lg	β-lactoglobulin
CCA	Chromogenic coliform agar
CCD	Central composite design
CFU	Colonies forming units
CI	Creaming index
EC	Electrical conductivity
EFSA	European Food Safety Authority
ENT	*Enterobacteriaceae*
ES	Emulsion stability
EVOO	Extra virgin olive oil
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FG	Fat globule
FGM	Fat globule membrane
GC-MS	Gas chromatography – mass spectroscopy
HHC	High – heat treated cream
HPP	High pressure processing
HS-SPME	Headspace solid-phase microextraction
HTST	High-temperature short-time

Abbreviation	Designation
LAB	Lactic Acid Bacteria
LPL	Lipoprotein lipase
MDD	Mean droplet diameter
MDG	Mono- and diglycerides of fatty acids
MFGM	Milk fat globule membrane
MRS	Man Rogosa and Sharpe agar
MUFA	Monounsaturated fatty acid
NHC	Nonheated cream
NIR	Near-infrared
O/W	Oil-in-water
PA/PE	Polyamide-polyethylene
PCA	Plate count agar
PL	Phospholipid
RBCA	Rose Bengal chloramphenicol agar
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
TAG	Triacylglycerol
TAM	Total Aerobic Mesophiles
TAP	Total Aerobic Psychrophiles
TG	Triglycerides
TSA	Trypticase Soy Agar
TSB	Tryptic Soy Broth
UHT	Ultra-high temperature

Abbreviation	Designation
VOC	Volatile compounds
VRBDA	Violet red bile dextrose agar
WHHC	Whipped high-heat-treated cream
WNHC	Whipped nonheated cream
W/O	Water-in-oil
YM	Yeasts and Moulds
450/5	450 MPa during 5 minutes
500/5	500 MPa during 5 minutes
600/5	600 MPa during 5 minutes
600/15	600 MPa during 15 minutes
ΔE^*	Total colour change

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Contextualization

The present work is divided into two parts. The first part comprises the work done in Aveiro, Portugal, regarding the effect of high pressure processing (HPP) on dairy cream's microbiology and physicochemical parameters. The second part concerns the work done during the Erasmus Program in Foggia, Italy, where it was investigated the optimum processing conditions of ultrasounds treatment in order to obtain a stable emulsion of olive oil and water, that would later be microbiologically stabilized by HPP in Aveiro.

Each part is divided into four chapters. Chapter I (Part I) comprises a comprehensive literature review regarding dairy cream, with special focus on its traditional processing technology, thermal pasteurization, as well as the introduction of an alternative non-thermal technology, HPP. As for Chapter I (Part II), it comprises a literature review regarding extra virgin olive oil, emulsions and ultrasound treatment. Following both chapters are the work objectives. Then, on Chapter II, a detailed description of the materials and methods used. The third chapter presents and discusses the results obtained for each set of experiments. On the fourth chapter are presented the main conclusions and future perspectives, followed by the list of the consulted literature in the scope of the present work.

PART I

CARRIED OUT IN AVEIRO, PORTUGAL

CHAPTER I - LITERATURE REVIEW

THIS SECTION COMPRISES A BRIEFLY COMPILED LITERATURE REVIEW REGARDING DAIRY CREAM AND NON-THERMAL TECHNOLOGIES

1. INTRODUCTION

1.1. Milk

Milk is mainly composed of water, fat, proteins, lactose and minerals (salts) (Bylund, 1995). Lactose, a disaccharide composed of glucose and galactose, is the predominant carbohydrate present in milk. The fermentation of lactose by lactic acid bacteria in cultured dairy products provides the desirable flavour and textural attributes in several dairy products, like cheese and yogurt (Walstra *et al.*, 2006; Simpson, 2012).

The fat present in milk is in the form of fat globules (FG), which are surrounded by a polar milk fat globule membrane (MFGM). More detailed information about the MFGM will be given in ***"section 1.2"***. **Figure 1** shows the main structural elements of milk. Triacylglycerols (TAGs) are the main lipid fraction in milk, accounting for 98% of the total lipids. Approximately 65% of the fatty acids (FAs) in milk fat are saturated, including 26% palmitic acid and 15% stearic acid and a significant amount of short- and middle-chain fatty acids are also present. These fatty acids and its breakdown products are important contributors to the flavour of many cultured dairy products (Simpson, 2012).

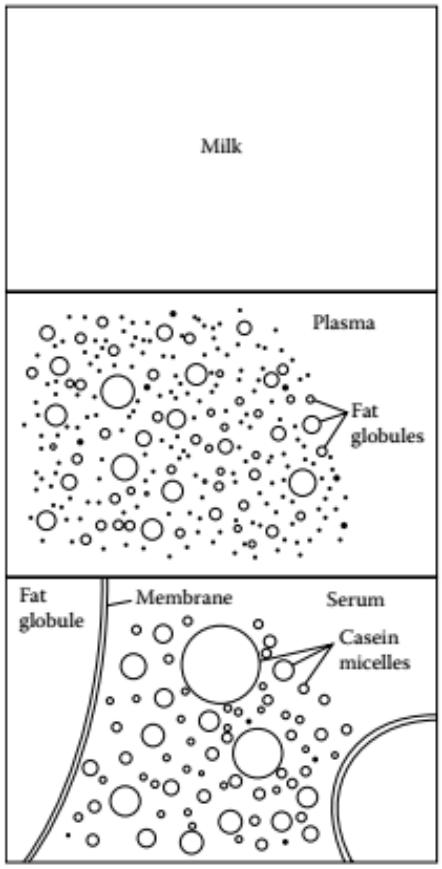

Figure 1 - Milk viewed at different extensions. Adapted from Walstra *et al.* (2006).

Caseins and whey proteins (mainly β-lactoglobulin (β-Lg) and α-lactalbumin (α-La)) are the two major classes of milk proteins. Caseins are hydrophobic proteins, hence dispersed in milk in the form of micelles, and are present as a mixture of four proteins: α_{s1}-, α_{s2}-, β-, and k-casein (Walstra *et al.*, 2006; Simpson, 2012), however, k-casein contains a hydrophilic portion which stabilises the structure (Cortes, 2010). In addition to the major protein fractions aforementioned, milk also contains numerous minor proteins, including a wide range of enzymes (e.g. lipases and phosphatases) (Cortes, 2010).

1.2. Milk fat

Milk is one of the rare biological fluids that exists as an emulsion in its native state (Lopez *et al.*, 2000). Milk fat is the most variable constituent of milk, varying both in proportion and composition, thus having a direct influence on its physical and chemical properties (Luquet, 1985). This variation can be related to changes in factors like cows breed, diet and lactation stage. The fat content can vary from about 3.0 to 6.0%, but typically ranges from 3.5 to 4.7% (Buchheim and Hoffmann, 2006). Milk lipids are very important since they confer distinctive nutritional, textural and organoleptic properties on dairy products, such as cream, butter, whole milk powder and cheese (Buchheim and Hoffmann, 2006). Those lipids are mainly composed of a mixture of TAGs and much smaller amounts of free FAs, mono-and diacylglycerols, phospholipids (PL) and sterols. Milk fat also contains pigments (e.g. carotene, which gives butter its yellow colour), supplies essential FA (linoleic, linolenic and arachidonic), fat-soluble flavouring compounds (Buchheim and Hoffmann, 2006) and acts as a solvent for the fat-soluble vitamins A, D, E and K (**Table 1**) (Cortes, 2010).

Table 1 - Main classes of lipids in milk. Adapted from Buchheim and Hoffmann (2006).

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Lipid class	Amount (%, w/w)
Triacylglycerols	98.3
Diacylglycerols	0.3
Monoacylglycerols	0.03
Free fatty acids	0.1
Phospholipids	0.8
Sterols	0.3
Carotenoids	trace
Fat - soluble vitamins	trace
Flavour compounds	trace

In native milk, fat is present in the form of small globules or droplets, dispersed in the milk serum as an oil-in-water (O/W) emulsion. These fat globules, which are surrounded by the MFGM, are not of uniform size, ranging in diameter from 0.1 and 20 µm, as shown in **Figure 2**.

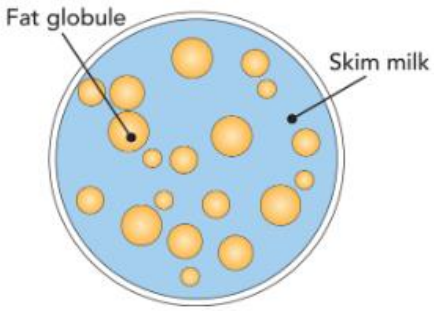


Figure 2 - Schematic representation of small globules of milk fat dispersed in the milk serum. Adapted from Bylund (1995).

The suspension of milk fat in the aqueous environment of milk is stabilized by the MFGM. These globules are relatively fragile, particularly when the fat is liquid, and can be readily disrupted by several conditions experienced in dairy processing operations. In particular, shearing, cavitation or turbulence, can damage the MFGM and cause physical rupture and sub-division of the globules (Buchheim and Hoffmann, 2006). The membrane is only 10-20 nm thick (Bylund, 1995), and represents about 2-6% of the fat globules' mass (Mezouari *et al.*, 2009). By surrounding the globules, it protects them against enzymatic degradation (lipolysis), and prevents them from coalescing into butter grains (Cortes, 2010).

The MFGM largely consists of polar lipids (mainly PL) which are closely associated with various specific proteins, being xanthine oxydase, butyrophilin and adipophilin the most abundant (**Figure 3**). These polar constituents present on the MFGM, allows the relatively high concentrations of milk fat and protein to coexist in the same solution (Deeth, 1997; Gassi *et al.*, 2008). Since PL of dairy origin are natural emulsifiers with functional properties, it can be used in the food, cosmetic and pharmaceutical industries (Gassi *et al.*, 2008).

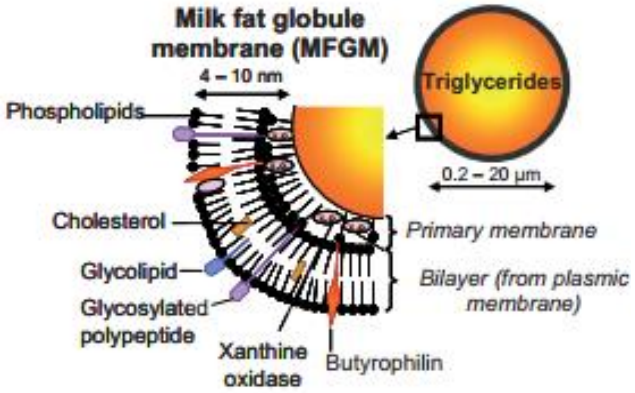


Figure 3 - Schematic representation of the milk fat globule membrane composition. Adapted from Gassi *et al.* (2008).

TAGs are the major fraction of neutral lipids in the MFGM, with over 400 different FAs identified in milk fat. This vast range and wide variety of FAs leads to a considerable number of possible molecular species of TAGs (Mezouari *et al.*, 2009). Some of the major FAs present in bovine milk, are listed in **Table 2**.

Table 2 - Major fatty acids in bovine milk. Adapted from Buchheim and Hoffmann (2006).

	Common Name	Composition		
		Typical %(w/w)	mol %	Range %(w/w)
4:0	Butyric	3.9	10.1	3.1-4.4
6:0	Caproic	2.5	4.9	1.8-2.7
8:0	Caprylic	1.5	2.4	1.0-1.7
10:0	Capric	3.2	4.3	2.2-3-8
12:0	Lauric	3.6	4.1	2.6-4.2
14:0	Myristic	11.1	11.1	9.1-11.9
14:1	Myristoleic	0.8	0.8	0.5-1.1
15:0	-	1.2	1.1	0.9-1.4
16:0	Palmitic	27.9	24.9	23.6-31.4
16:1	Palmitoleic	1.5	1.4	1.4-2.0
18:0	Stearic	12.2	9.8	10.4-14.6
18:1 *cis*	Oleic	17.2	13.9	14.9-22.0
18:1 *trans*		3.9	3.2	
18:2	Linoleic	1.4	1.1	1.2-1.7
18:2 conj	Conjugated Linoleic acid	1.1	0.9	0.8-1.5
18:3	α Linolenic	1.0	0.8	0.9-1.2
	Minor acids	6.0	5.1	4.8-7.5

The FA composition associated with MFGM-TAGs is characterized by their high proportions of long-chain FA C16:0, C18:0 and C18:1, and low contents of C14:1, C16:1 and C18:2 (**Table 2**) (Mezouari *et al.*, 2009). Because TAGs account for about 98% of the total fat, they have a major and direct effect on milk fat properties, for example hydrophobicity, density and melting characteristics (Jensen and Newburg, 1995).

Phospholipids in milk account for only 0.8% of milk lipids. However, they are important for MFGM structure due to their amphiphilic properties, that allows them to stabilise emulsions and to form micelles and membranes (Jensen and Newburg, 1995). In bovine milk, about 55-70% of the phospholipids are associated with fat globules, whereas the rest remain in the aqueous phase, associated with protein/membrane fragment material in solution, rather than still attached to the MFGM (Le *et al.*, 2014). During milk processing, phospholipids are partitioned differently from the neutral lipids (**Table 3**). When the hole milk is separated, the phospholipids tightly bound to the MFGM go into the cream with the neutral lipids, while the phospholipids associated with the protein/membrane fragments in the aqueous phase are retained in the skim milk. Furthermore, during cream churning for butter making, the MFGM is broken and a greater proportion of the phospholipids than the neutral lipids from the cream is mainly found in

the buttermilk, which is the by-product of butter manufacture, leading to a higher ratio of phospholipid to the total fat in buttermilk (Buchheim and Hoffmann, 2006; Gassi *et al.*, 2008). Moreover, heat treatment of cream may modify MFGM's composition and its surface properties (Gassi *et al.*, 2008). It has been reported that heat treatment causes denaturation of the MFGM proteins and interaction with whey proteins (β-Lg and α-La). Part of the original MFGM that remains on the globule is insufficient to cover the new surface, thus casein semi-intact micelles and micellar fragments completely involve the new surface, avoiding the coalescence of fat globules (Luo *et al.*, 2014). More detailed information about the effects of heat treatment on cream constituents will be focused on ***"section 1.3.2."***.

Table 3 - Approximate phospholipid content of different dairy products. Adapted from Buchheim and Hoffmann (2006).

Product	Whole milk	Skim milk	Cream (40% fat)	Buttermilk
Total fat (%, w/w)	4	0.06	40	0.6
Phospholipids (%, w/w)	0.035	0.015	0.21	0.13
Ratio (g PL/100 g total fat)	0.9	25	0.5	22

Even though fat globules are the largest particles in milk, they are the lightest (density at 15.5 °C = 0.93 g/cm^3), thus they tend to rise to the surface when milk is left to stand in a vessel for a while, forming de cream layer. Reducing the size of the milk fat globules, by a process called homogenization, prevents this layer to be formed (Banks, 1993). However, if the final goal is to produce cream and its products, this homogenization process is not desirable.

1.2.1. Emulsion instability and milk separation

Emulsions may destabilize via several different mechanisms, and this instability can have a physical or chemical nature. Chemical instability results from an alteration in the chemical structure of lipid molecules due to oxidation or hydrolysis, while physical instability results from an alteration in the spatial distribution or structural organization of the globules. The mechanisms responsible for physical instability of emulsions can be divided into two categories: gravitational separation and droplet aggregation (**Figure 4**) (Buchheim and Hoffmann, 2006), as follows:

(1): Gravitational separation: involves the movement of emulsion droplets due to the fact that they differ in density from the surrounding liquid. If the droplets have a lower density than the surrounding medium, they tend to move upwards, in a process called *creaming*. Conversely, if they have a higher density than the surrounding medium, they tend to move downwards under the influence of a gravitational force, and sedimentation occurs (Buchheim and Hoffmann, 2006).

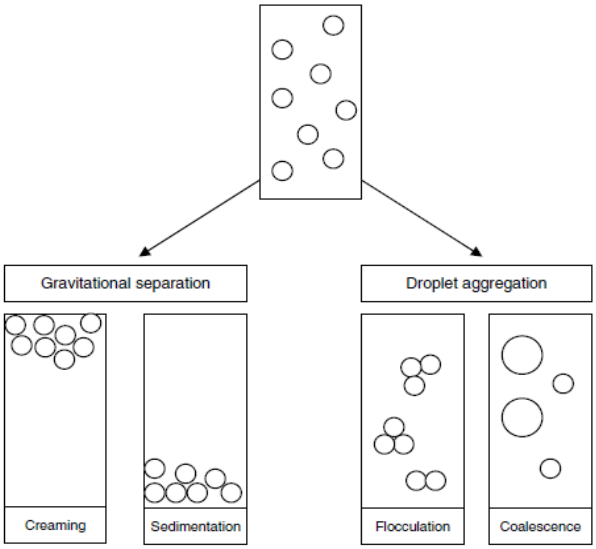


Figure 4 - Schematic overview of emulsion's types of instability. Adapted from Buchheim and Hoffmann (2006).

(2): The droplet aggregation: involves the interaction between emulsion droplets, by flocculation or coalescence. The first occurs when two or more droplets come together to form an aggregate but retain their individual integrity, while the second mechanism arises from the rupture of the film that separates the droplets and two or more small droplets aggregate to form a larger one (Tobin *et al.*, 2015).

Milk's fat separation can be significantly accelerated through the application of a centrifugal force, which is the principle of separation (skimming) of milk in industrial practice. The objective of centrifugal separation is to achieve the lowest fat content possible in skimmed milk, while concentrating the fat on the cream phase.

1.3. Cream

Despite contributing with only 2% to the national production of dairy products, cream is one of the most important dairy products (Simpson, 2012). The word "cream" has for a long time been associated with a premium product, since milk fat develops a unique flavour and its properties makes it also a preferred ingredient in many other foods (Hoffmann, 2016).

Generally, cream is considered the fluid milk product comparatively rich in fat, in the form of an emulsion of fat-in-skimmed milk, obtained by physical or mechanical separation of milk (Deosarkar *et al.*, 2016). The physical and chemical properties of cream are deeply influenced by the MFGM, as well as the concentration and state of fat globules, which have a marked effect on the rheology and physical state of cream. The non-fat milk solids such as proteins, salts, added emulsifiers and stabilizers also play an important role in the cream

properties. In addition, temperature also impacts cream properties, affecting the state of different lipid components, and the physical handling of cream like pumping, aeration and agitation, can cause globules to disrupt or agglomerate (Smiddy *et al.*, 2009). Creams may differ in fat content, but also in the degree of fat dispersion, which is strongly affected by homogenization during processing. Both characteristics determine the functional, rheological, and sensory properties of the resulting product (Hoffmann, 2016).

Cream and cream products are now readily used in many forms and for many purposes, like serving as the primary raw material for the manufacture of table butter. In the European Union, for example, about 30% of the available milk fat is processed into butter (Hoffmann, 2016). Despite all its potential applications, creaming can also be undesirable in many dairy products (e.g., liquid/beverage milk, concentrated milks), being prevented by a process known as homogenization (Simpson, 2012). Homogenization at pressures typically in the range 10-30 MPa has been used for more than 100 years in the dairy industry do reduce milk fat globule size and, hence, to prevent creaming on storage. It is used in the production of many dairy products, such as milk, yoghurt, ice cream and cream liqueurs, improving their texture, taste, flavour and shelf-life (Banks, 1993).

1.3.1. Types of cream and cream products

Creams are usually classified according to their fat content (g/100g), to the heat treatment to which they were subjected (processing method) and also according to their final use (Luquet, 1985). **Table 4** shows some of the commercially available creams and its applications (Budhkar *et al.*, 2014). The most typical of all cream products is whipping cream, which has a fat content of 30-40%, and requires no complicated preparation, just careful handling before whipping. A well homogenized cream, with the lowest legally permitted fat content (10-12%), is preferably used as coffee whitener. While this coffee cream is added just before enjoying the coffee, double cream (45-50% fat) is added already during the manufacture of another kind of beverage, cream liqueur. Cultured or sour creams have a fat content ranging from 10-40% and its manufacturing process is largely equivalent to that of other fermented products (Hoffmann, 2016). Cream can also be churned, leading to butter and buttermilk, being the latter similar in composition to skim milk (Walstra *et al.*, 2006).

Table 4 - Commercially available cream, its fat content and applications. Adapted from Budhkar *et al*. (2014).

Cream type	Fat content (% by weight)	Applications
Half-cream or singles cream	10 - 18	• As pouring cream for use in desserts and beverages; • As breakfast cream poured over fruit and cereals; • Used industrially as an ingredient of canned soups and sauces.
Coffee cream	Up to 25	To give an attractive appearance to coffee with appropriate modification in flavour.
Cultured or sour cream	<25 normally (occasionally up to 40)	In confectionery, and in meat and vegetable dishes.
Whipping cream	30 - 40	For toppings and fillings for baked goods
High-fat creams (plastic cream)	70 - 80	For ice cream manufacture

1.3.1.1. Whipping cream

Whipped cream is valued by consumers for its taste and texture, and its created by beating air into cream until a stiff-foamed product is produced (Smiddy *et al*., 2009). As mentioned before, whipping cream should have a fat content between 30 and 40%, and should be pasteurized without homogenization, since upstream homogenization would cause an undesirable increase in the free-fat content. Whipping cream tolerates homogenization only at a relatively low pressure in order to retain acceptable whipping properties (Hoffmann, 2016). Besides tasting good and keeping well, whipping cream must also have good ‘’whippability’’, being easy to whip and producing a fine cream foam with an increased volume (overrun). The foam must be firm, stable and must not be susceptible to syneresis. Moreover, good whippability depends on the cream having a sufficiently high fat content. Whipping cream with 40% fat, is usually easy to whip, however the whippability decreases as the fat content drops to 30% or below (Hoffmann, 2016).

During the first stage of the whipping process, air is beaten into the cream, resulting in a coarse foam. The air bubbles formed (~ 150 µm diameter) in the foamed cream rapidly become covered by milk proteins, which stabilise them against collapse. Subsequent whipping reduces air bubble size, causing milk lipid globules to displace some of the

proteins from the bubble interface. This fat adsorption involves the partial loss of the fat globule membrane (FGM) and the spreading of fat at the bubble interface, creating an air-lipid interface. Thus, if the air bubbles collapse, it will lead to partially uncovered globules, which are extremely susceptible to partial coalescence (Smiddy *et al.*, 2009).

Each manufacturing process for different milk and cream products must consider the quality of raw milk. However the demands can vary according to the resulting product and its purpose (Hoffmann, 2016). Although the processing of long-life coffee cream is characterized by severe homogenization and heat treatment, the original or heat-induced sensorial deviations are partially masked after addition to the coffee beverage, as mentioned before. On the contrary, whipping cream has to be produced carefully with little thermal and mechanical input, and the quality of the raw milk for its production is very important since this cream is consumed for its pure flavour (Buchheim and Hoffmann, 2006; Hoffmann, 2016).

The appearance of psychrotrophic bacteria, known to produce extracellular lipases and proteinases, can be a consequence of a prolonged refrigerated storage of raw milk. Since these enzymes can survive pasteurization or even ultra-high temperature (UHT) heating, they might be responsible for the emergence of rancid and tallowy flavour, or even physical changes (Deosarkar *et al.*, 2016; Hoffmann, 2016). After separation of raw milk at about 55 °C, the microbial load of cream is heavier than that of skim milk, since the high fat content of cream protects microbes during heating (Deosarkar *et al.*, 2016; Hoffmann, 2016). Cream pasteurization is commonly carried out at 80 °C. Subsequently, the cream is cooled, aseptically packaged and distributed, having a shelf-life of <3 weeks at refrigeration temperature. The need to extend the shelf-life of creams has led to the production of UHT whipping cream, which, after the optional addition of stabilizers, is heated at >135 °C for a few seconds (Deosarkar *et al.*, 2016). At least 110 °C for 10 s is essential for the inactivation of mesophilic spores and to achieve a 3 weeks shelf-life at 10 °C. More information about cream processing is discussed in "***section 1.3.2***"

Warm cream contains liquid fat, which makes whipping impossible. Cream for whipping must be therefore stored at low temperatures (4-6 °C) over a relatively long period of time (ripening time) to obtain proper fat crystallization , since the transformation of the original O/W emulsion into a stable foam requires that part of the fat to be solid (Bylund, 1995; Buchheim and Hoffmann, 2006). A critical situation can occur in the cooling section because the fat globules are very sensitive to mechanical damage during crystallization (Hoffmann, 2016). Due to the partial or complete loss of the protective

membrane, the exposed fat is hydrolysed to fatty acids by both indigenous and bacterial lipases, inducing rancid taints (Buchheim and Hoffmann, 2006).

During transport and storage cream temperature is crucial for its physical stability, since even a brief warming to ≥ 30 °C favours creaming during subsequent storage at 20 °C and may lead to a distinct thickening after cooling before whipping. In addition, continuous cooling during the whole shelf-life delays creaming, avoids destabilization and sensorial changes, also resulting in increased foam volume, but a longer whipping time (Buchheim and Hoffmann, 2006).

1.3.2. Cream processing: Heat treatment

Food processing is necessary to transform raw animal or plant materials in order to produce consumer-ready products, stabilizing these food products by preventing or reducing negative changes in quality. Without these processes, it wouldn't be possible to store food, neither from time of plenty to time of need nor for transportation (Hogan and Kelly, 2005).

Concerning the type of processing received, creams can be either raw, i.e, undergo no heat treatment, pasteurized, sterilized or ultra-high-temperature treated cream (Smiddy *et al.*, 2009; Deosarkar *et al.*, 2016). A typical flow sheet for manufacture of sterilized cream in can is shown in **Figure 5**.

Traditionally, most of the cream produced for retail consumption and industrial use is pasteurized (Early, 1998). This heat treatment is necessary to destroy vegetative microorganisms that may be pathogenic or cause spoilage, and to inactivate enzymes, thus extending creams shelf-life. Proteolytic enzymes may produce bitter peptides and also cause coagulation, whereas lipolytic enzymes break down the lipids, producing fatty acids which give a rancid flavour (Deosarkar *et al.*, 2015; Y. Kotilinga Reddy, 2018).

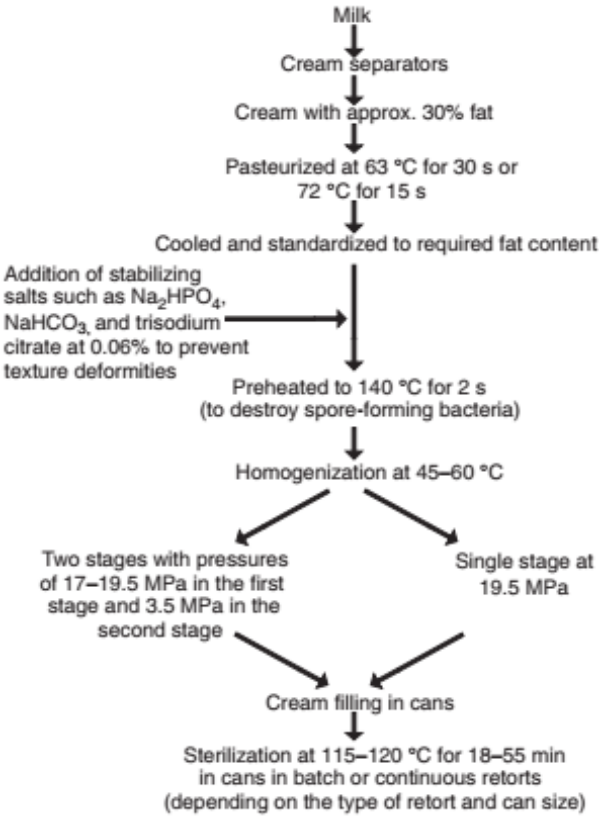


Figure 5 - Flow sheet of typical manufacturing process for sterilized cream. Adapted from Budhkar et al. (2014).

The effect of heating depends mainly on the intensity of the treatment, i.e., the temperature and duration of heating. Usually, to pasteurize cream, either high-temperature short-time (HTST) pasteurization or UHT sterilization is applied. The fat content of cream protects microbes during heat, thus cream needs a more severe heat treatment than milk (Deosarkar *et al*., 2015; Y. Kotilinga Reddy). Currently, HTST treatment of creams is most used by commercial creameries for sterilization (Budhkar *et al.*, 2014). Most vegetative cells, including pathogens, yeasts and moulds, are inactivated by pasteurization and UHT treatment, however, it does not necessarily produce a sterile product, since some thermoduric bacteria, including spore-forming thermoduric *Bacillus* spp., can survive both treatments (Early, 1998; Smiddy *et al.*, 2009).

The indigenous milk enzymes, lipoprotein lipase and proteinase, cause lipolysis and proteolysis, respectively, during storage (Early, 1998; Smiddy *et al.*, 2009). Although lipases are largely inactivated by pasteurization, considerable plasmin activity remains even after UHT treatment (Bastian and Brown, 1996; Kosinski, 1996). Since bacterial lipases and proteinases are particularly heat-stable and can survive UHT treatment, their production must be prevented in UHT cream (Smiddy *et al.*, 2009). Efficacy of heat

treatment must be checked by testing for phosphatase. However, the use of this test can be problematic, since this enzyme can be reactivated during storage (Budhkar *et al.*, 2014).

Depending on the food matrix, conventional thermal pasteurization treatment may not be the most adequate processing, once it relies on extensive heat treatment, where heat slowly penetrates the core of the product, making the cooling process also slower. This kind of process could also be responsible for considerable changes on the product's quality, developing off-flavours and destruction of vitamins and other nutrients (Hogan and Kelly, 2005). However, in spite of these disadvantages, thermal processing remains the dominant method for food preservation, due to its high availability, low cost, high productivities and effectiveness on reducing microbial levels (Lopes *et al.*, 2016).

Since consumers consider sensory characteristics such as texture, flavour, aroma, shape and colour extremely important attributes of a food product, and that nowadays there is an increasing demand for minimally processed products, with extended shelf-life, new preservation technologies, especially non-thermal, that retain or create desirable sensory qualities or reduce undesirable changes in food due to high temperatures, are being tested and developed (Hogan and Kelly, 2005).

The application of high pressure processing (HPP) has shown considerable potential as an alternative technology to heat treatments, both in terms of assuring safety and quality attributes of minimally-processed food products (Hogan and Kelly, 2005). More details on this potential technology will be discussed in ***"section 1.4"***.

Gassi *et al*. (2008) studied creams with three different heat treatments: low (88 °C for 80 s), medium (94 °C for 80 s) and high (70 °C for 2 h then 88 °C for 30 s and finally, 94 °C for 80 s, in three successive steps). Regarding protein content, they concluded that the total protein content in cream significantly decreased from low heat to medium heat and to high heat-treated cream (18.21, 17.46 and 16.83 g/kg, respectively). Concerning the particle size distribution of creams, on the high heat-treated it ranged from 0.02 µm to 200 µm, with three peaks centred at about 0.5 µm, 4 µm and 45 µm (**Figure 6**). Due to the addition of sodium dodecyl sulphate (SDS), an anion detergent that dissociates the aggregates of fat globules, the peak at about 45 µm was highly decreased, confirming that it corresponded to fat globules aggregates. This peak of fat aggregates appeared with the highest heat treatment. The measurement of individual fat globule diameter, which ranged from 0.4 to 70 µm, was possible due to their dissociation with SDS. As for low heat-treated cream, since globules were well individualized, the addition of SDS did not change their size distribution, which ranged from about 0.03 µm to 13 µm. Thus, no aggregates of fat

globules were present in these creams (**Figure 6**). Regarding medium heat-treated creams, some larger fat globules, i.e. with diameter >20 µm were found, in the presence of SDS.

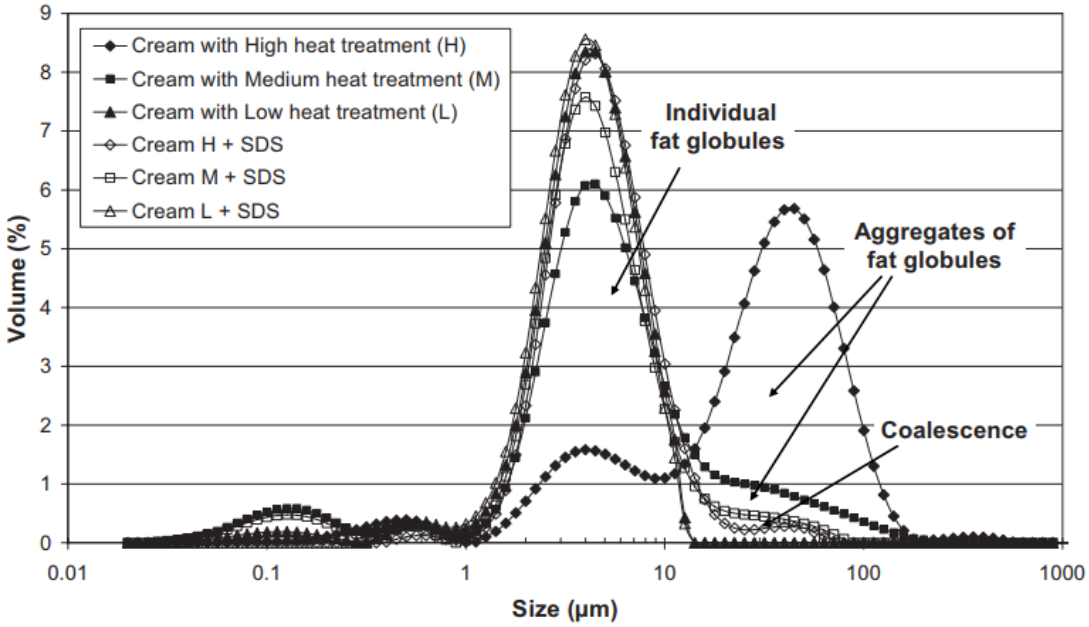


Figure 6 - Particle size distributions of low heat-treated cream (L), medium heat-treated (M) and high heat-treated (H). Adapted from Gassi et al. (2008).

High heat-treated creams, compared with low heat-treated creams have higher viscosity, which can be explained by fat globules' aggregation (Gassi *et al.*, 2008). According to Ye, Singh, Taylor, and Anema, (2004) the heat treatment of whole milk causes the association of whey proteins (mainly β-Lg and α-La) and caseins (mainly κ-casein) to the MFGM surface. However, the mechanisms by which these proteins interact with the fat globule are still controversial. These associations begin at relatively low temperatures (60-65 °C) and increase with increasing temperature and heating time (Gassi *et al.*, 2008).

Figure 7 shows a schematic representation of MFGM composition in raw milk or after low heat treatment (**Figure 7A**) and after a severe heat treatment, with the adsorption of aqueous phase proteins on the MFGM (**Figure 7B**).

These modifications on MFGM's composition, consecutive to a heat treatment, involves changes in interfacial properties, which might result in the aggregation of fat globules. The authors concluded that this aggregation of high heat-treated fat globules could explain variations in viscosity and stability of sweet, industrial creams before churning (Gassi *et al.*, 2008).

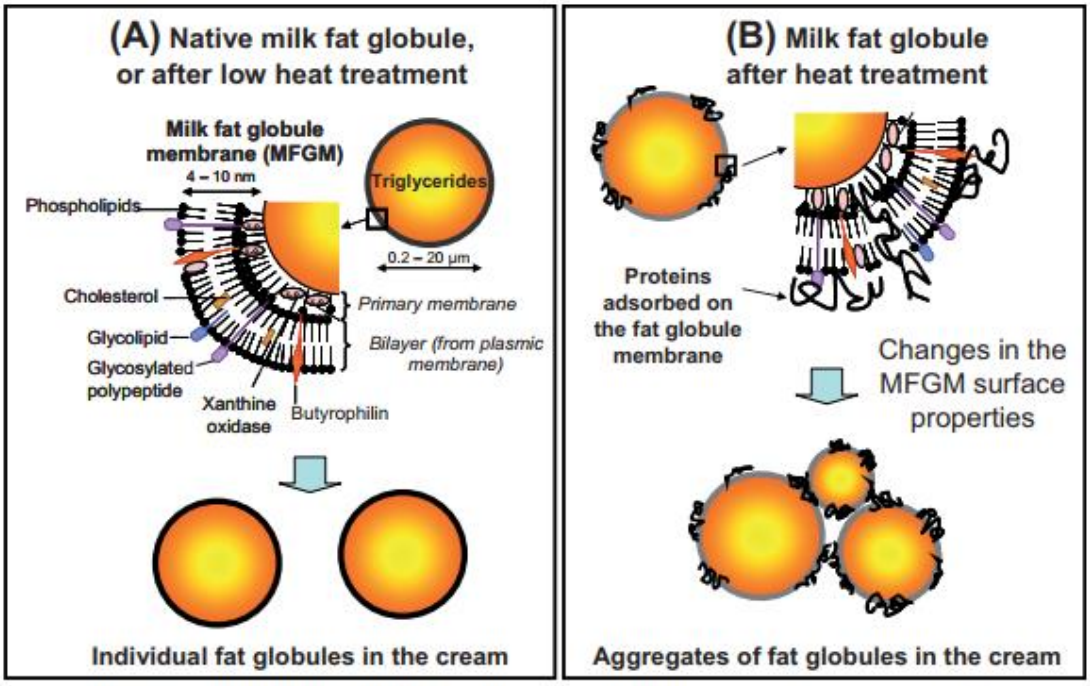


Figure 7 - Changes in MFGM composition as a function of the thermal treatment applied to the cream. Schematic representation of MFGM composition **(A)** in raw milk or after low heat treatment and **(B)** after high heat treatment, inducing the aggregation of fat globules. Adapted from Gassi *et al.* (2008).

1.3.3. Quality of cream and sensorial analysis

Cream quality depends on the handling of milk from which it is prepared and its physicochemical and microbiological properties (Budhkar *et al.*, 2014). The use of poor-quality raw milk contributes to most of the difficulties experienced during cream preparation. In order to avoid these difficulties, it is particularly important that raw milk is unadulterated, free of taints, antibiotics, blood or visible sediments (Varnam and Sutherland, 1994; Smiddy *et al.*, 2009).

1.3.3.1. Microbiology

Cream production is essentially a dairy operation, however cream may still be produced on farms and sold directly to the public. Hygiene is very variable, and high counts of bacteria, yeasts, and moulds may be found in 2 or 3 days, rendering in a correspondingly short shelf-life (Wilbey, 2002). And so, milk production on the farm should be done in

utmost hygienic manner. Milk should also be handled carefully since excessive agitation or pumping can cause air to be drawn into the milk. This can damage the MFGM resulting in free fat that may coalesce or churn, making separation difficult (Smiddy *et al.*, 2009).

Despite the fact that vegetative cells may be killed by subsequent heat treatment, spores and organisms such as *B. cereus* can survive and cause subsequent spoilage of milk (Budhkar *et al.*, 2014). The high fat content of cream also imply problems due to lipolytic enzymes of psychrotrophic bacteria, thus the refrigerated storage period for the raw milk must be carefully controlled and should not exceed 24 hours (Varnam and Sutherland, 1994; Smiddy *et al.*, 2009).

A simple farmhouse pasteurization (heating to 65 °C and holding for 30 min) will reduce the bacterial count to about 1% or less of the original, however, unless it is followed by rapid cooling to 5 °C, it will have little ultimate effect on keeping its quality (Wilbey, 2002).

Common taints found in farm-produced cream include sour, rancid, cheesy, stale, bitter, putrid, and yeasty. These problems are always associated with high microbial counts, being *Pseudomonas* the predominant organism responsible for the dominant taint (Wilbey, 2002). In bad cases of spoilage, gas may be formed, usually by lactose-fermenting yeasts and mould growth may also be visible on the surface of cream. Souring by lactic acid bacteria may repress putrefactive organisms, however their activity can stimulate yeasts and moulds. Sweet curdling may be caused by proteolytic enzymes produced by aerobic spore-formers, which can also be responsible for bitterness (Wilbey, 2002).

Farms' technological proficiency has become largely similar to that of the dairies, except for its scale operation. Hence, the microbiological quality of farm-produced cream should not differ significantly from that retailed by a creamery. At an industrial scale, apart from cultured or soured cream, the entire process of manufacturing, packaging, and cream distributing is, from a microbiological point of view, a matter of preventing contamination and keeping the growth of the few organisms that are present to a minimum (Wilbey, 2002).

1.3.3.2. Physicochemical properties and sensorial analysis

One of the important factors that determines consumer acceptability of cream is the visual assessment of its 'body' or viscosity. The viscosity of the resulting cream, can be affected by factors associated with milk (e.g. the TAG content), its processing and storage (Smiddy *et al.*, 2009). Creams with high fat content tend to be more viscous, moreover, during storage, the continuous flocculation of fat globules and the gradual accumulation of

casein micelles onto globule surfaces and bridging at points of contact, strengthens the structure contributing to an increase in viscosity. The rate of this increase, is dependent on homogenization pressure, fat content and heat treatment (Varnam and Sutherland, 1994). Besides increasing the viscosity, homogenization also increases the potential for light-induced rancidity, due to the increased surface area. Cream's characteristic flavour and aroma are derived primarily from constituents of the fat phase, however there is also a contribution from constituents of the aqueous phase and the MFGM (Varnam and Sutherland, 1994).

Schlutt *et al.* (2007) studied the influence of mechanical, as well as, thermal treatment on the sensory profile of a full-fat cream sample. Thus, a non-heated and a high-heat-treated cream samples were presented to a trained sensory panel, before and after whipping, respectively. In a preliminary experiment, the orthonasal aroma of the four cream samples (nonheated cream - NHC, high-heat treated cream - HHC, whipped nonheated cream - WNHC and whipped high-heat treated cream - WHHC) were evaluated. The assessors were able to distinguish between samples NHC and HHC, as well as between samples WNHC and WHHC, indicating that both thermal treatment and whipping procedure induced changes in the orthonasal aroma of cream.

Furthermore, to investigate the individual aroma profiles of the four cream samples, aroma profile analyses were performed.

As shown in **Figure A1** (**Appendix A**), the NHC **(A)** exhibited a rather weak overall aroma, centering mostly around buttery, creamy and a slightly metallic aroma. By comparing it to the WNHC sample **(C)**, it is noticeable that whipping the cream, led to a more intense creamy, buttery, fatty and sweaty aromas. The overall aroma intensity of the cream was strongly enhanced when the sample was thermally treated **(B)**. The strongest effects were observed in the intensity of creamy, buttery, popcorn-like and sulfury aromas. Moreover, whipping this thermally treated sample induced an additional increase of the creamy descriptor, since maximum intensity of 2.8 for creaminess was detected. In addition, the WHHC sample **(D)** was described as more coconut-like and buttery than the heat-treated cream prior to whipping. This confirms that the mechanical treatment also affects the sensory perception of cream.

Based on this sensory evaluation, the authors concluded that the thermal treatment is a key step in enhancing the perceived creamy flavour. The evaluation also revealed that the WHHC was the creamiest product.

1.4. High Pressure Processing (HPP)

In order to inactivate undesirable microorganisms and give an acceptable shelf-life, food is usually preserved by heat treatment. However, high temperatures can adversely affect flavour and the nutritional value of some foods, through Maillard browning or destruction of vitamins, for instance (Voigt *et al.*, 2015).

Nowadays, there is an increasing demand for more natural products, with minimum processing and with an extended shelf-life. To meet these new challenges of the food industry, new preservation technologies, especially non-thermal treatments such as HPP, have been tested and developed (Evert-Arriagada *et al.*, 2014).

HPP is a non-thermal food processing technology that makes use of elevated hydrostatic pressure (about 400-600 MPa) to induce the pasteurizing effect, denaturing various enzymes and inactivating pathogenic and vegetative microorganisms, depending on the pressure applied, thus ensuring food safety (Elamin *et al.*, 2015). Therefore HPP may represent one of the most promising possibility for preserving and preparing food products with improved functional and microbiological properties (Voigt *et al.*, 2015).

Food safety and shelf-life are often closely related to microbial quality, however other phenomena such as biochemical and enzymatic reactions and structural changes can also significantly influence consumers perception of food quality. Conventional thermal sterilization processes can induce changes in the product's quality, such as off-flavour generation, textural softening and destruction of colours and vitamins (Hogan and Kelly, 2005). However, as stated already, HPP, unlike thermal treatment, at moderate pressures, can inactivate pathogenic/spoilage microorganism while leaving most attributes of food quality intact (Hendrickx, 2001). These advantages have led to high consumer acceptability of HPP treated products like guacamole, juices, oysters, ham, fruit jellies and jams, pourable salad dressing, salsa, poultry and rice products, which are available in supermarkets (Chawla *et al.*, 2011). **Table B1** (**Appendix B**) shows some high pressure treated products available in international markets.

The behaviour of foods under the effect of HPP can be explained by two main principles, the Le Chatelier's and the isostatic principles. The first one states that any change made in an equilibrium (chemical reaction, phase transition or modifications of molecular configuration) accompanied by a volume decrease is compensated by a pressure increase, and vice-versa. The second one claims that regardless of the size and/or geometry of the food, pressure is transmitted uniformly and simultaneously in all directions, and that

after decompression, the material returns to its initial shape (Elamin *et al.*, 2015). This is another benefit of HP-processed food when compared to heat-processed food, where different thicknesses lead to overheating at the surface and to inadequate temperatures in the centre of the product, which does not ensure food safety (Voigt *et al.*, 2015). However, one of the limitations of HPP is that the food product must contain water, since the entire process is based on compression (Chawla *et al.*, 2011).

Concerning HPP feasibility against vegetative cells, it is based on the interruption of cellular functions that are essential for reproduction and survival of microorganisms. Accordingly, HPP is responsible for changing the microorganism's membranes, resulting in leakage of the inner cell content and interference on nutrient uptake mechanisms. Additional damaging events include extensive solute loss during pressurization, protein denaturation and enzymatic inactivation. Due to the rigidity of teichoic acids present on the peptidoglycan layers of Gram-positive bacteria, its inactivation normally requires more intense pressure treatments than Gram-negative bacteria (Mújica-Paz *et al.*, 2011). More detailed information about the HPP effect on microorganisms will be given in ***"section 1.4.1"***.

The application of HPP technology to food products was first reported by Hite (1899), who proved that it was possible to extend raw milk shelf-life for 4 days after pressure treatment of 1 hour at 600 MPa at room temperature, since the milk suffered a microbial reduction between 5 to 6 logarithmic cycles (Hite, 1899).

Despite the fact that the discovery of HPP food processing and its effects on microbial inactivation was unleashed in 1899 by Hite and other researchers, this technology only started to draw attention after almost 100 years when a commercial HPP equipment came out in Japan in 1990 for the first time, together with the commercialization of the first food product processed by HPP, a fruit jam. Mitsubishi Heavy Industries was the first to manufacture a HPP vessel devoted to food processing (Elamin *et al.*, 2015). After the Japanese revolution, HPP equipment technology was gradually established in other countries and the number of equipments and products processed by this technology has increased (**Figure B1 – Appendix B**), mainly due to consumer requirements for fresher, tastier and minimally processed foods (Huang *et al.*, 2013; Elamin *et al.*, 2015).

Currently, HPP is a well-established technology, being widely used as a non-thermal food pasteurization procedure (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). Several companies are focused on the manufacture, development and innovation of HPP equipments (Elamin *et al.*, 2015). However, the high cost, lack of investment in emerging

technologies and commercial equipment capable of operating with minimal disruptions and processing large amounts of food were the causes for the slow evolution of this technology in the food industry (Torres and Velazquez, 2005). This disadvantage can be less meaningful with a gradual increase in the volume of foods processed by HPP, advancements in HPP equipment and optimization of process parameters (Gupta and Balasubramaniam, 2012).

1.4.1. Effects of high pressure on microorganisms

The effectiveness of any food preservation technique is primarily evaluated based on its ability to eradicate pathogenic microorganisms present, thus enhancing the product's safety. Only after ensuring the absence of these pathogenic microorganisms, that a secondary objective, regarding the inactivation of spoilage microorganisms, is taken into account to improve food's shelf-life. These spoilage microorganisms in food can produce unacceptable changes in taste, odour, appearance and texture (Hogan and Kelly, 2005).

Microorganisms are an heterogenous group of organisms, which are capable of growing at temperatures from well below freezing (extreme psychrophiles) to above 100 °C (extreme thermophiles). As with heat, large differences in pressure resistance are evident among various strains of the same species. The bacteria growth stage is also important in determining pressure resistance, with cells in the stationary phase being more resistant than those in the exponential phase (Hogan and Kelly, 2005).

L. monocytogenes and *Staphylococcus aureus* are probably the two most intensively studied species in terms of use of HPP. *L. monocytogenes* is a Gram-positive rod of concern when it comes to acidified and other foods, such as dairy products and ready-to-eat meats, since it is a foodborne bacterium that requires particular care for processing and storage because it is moderately heat resistant and can grow anaerobically under refrigeration. As for *S. aureus*, it appears to have high resistance to pressure (Erkmen O, 1997; Hogan and Kelly, 2005).

It is well stablished that spores are the most pressure-resistant life forms known. In general, only very high pressures, >800 MPa, can kill bacterial spores around ambient temperatures. Alternatively, other processing methods, applied in combination with HP, can be effective for bacterial spore elimination, by achieving a synergistic effect. In particular, HPP at elevated temperatures (>90 °C), is very effective in eliminating bacterial spores in foods. The most heat-resistant pathogenic bacterium is *Clostridium botulinum* and its spores are also among the most pressure-resistant microorganisms known.

Moreover, another spore-forming bacteria of concern is *B. cereus*, due to its anaerobic nature and very low rate of lethality (Hogan and Kelly, 2005).

Instead of combining heat and pressure to enhance killing bacterial spores, an alternative is to first cause bacterial spore germination (e.g. 50-300 MPa) and then use HPP to kill the much more pressure-sensitive vegetative cells. Process temperatures in the range of 80-110 °C in conjunction with pressures of about 600 MPa, have been used to inactivate spore-forming bacteria, such as *B. cereus* (Van Opstal *et al.*, 2004). Cycling treatments, where spores are exposed to alternating low and high pressures, or alternating cycles of pressurization and depressurization, are also of interest for sterilization process (Hogan and Kelly, 2005).

1.4.1.1. Factors influencing microbial sensitivity to high pressure

As mentioned before, the pressure resistance of microorganisms varies considerably depending on species, strain, stage of growth and food composition. In order to optimize treatments to assure microbiological safety, some factors that can affect the response of microorganisms to pressure, need to be taken into account. These factors include, pH of the food where the microorganism is likely to grow, water activity (a_w), temperature, pressure and holding time. The pH of the food is one of the main factors affecting microorganisms' growth and survival. All microorganisms have a pH range in which they can grow and an optimal pH at which they grow best. If the pH of a food is not optimal for a particular species, it will not only enhance its inactivation during treatment, but also inhibit outgrowth of sub lethally injured cells. Generally, at acidic pH values, extents of pressure-induced inactivation will be enhanced and recovery of sub lethally injured cells inhibited (Hogan and Kelly, 2005). While many HPP are performed at ambient temperature, increasing or, to a lesser extent, decreasing temperature, has been found to increase the inactivation rate of microorganisms during HPP. Also, there is a minimum critical pressure below which microbial inactivation by HPP will not take place regardless of process time (Hogan and Kelly, 2005).

Given that water in the liquid state is essential for the existence of all living organisms, lowering the amount of water available (water activity) will also significantly influence the growth of food spoilage or food-poisoning organisms that may be present in the raw material or introduced during processing. Reducing the a_w appears to protect microbes against inactivation by HPP. On the other hand, lowering a_w inhibits the recovery of sub

lethally injured cells. Consequently, it may be difficult to predict the effect of water activity on microbial inactivation by HPP (Hogan and Kelly, 2005).

1.4.2. Applications of HPP on dairy creams

Dumay *et al.* (1996) have studied the effect of HPP on the physical stability and flow behaviour of thermally pasteurised and sterilized dairy creams. Before pressurization treatment, those creams exhibited well dispersed fat globules, with diameters between 0.5 and 10 µm (**Figure 8**), rarely presenting FG aggregates (60 to 80 µm). A preliminary dilution of pasteurized or sterilized creams in SDS before laser diffractometry measurements did not significantly change the size distribution profiles, indicating the absence of FG aggregates in liquid creams.

Concerning the rheological properties of creams, they presented a slightly shear-thinning (pseudoplastic) behaviour. By comparing both creams, it was observed that while pasteurized cream displayed low viscosity (7.5 ± 0.63 mPa.s), sterilized cream displayed more variable and higher viscosity (varying between 10 and 23 mPa.s), in agreement with the smaller droplet size.

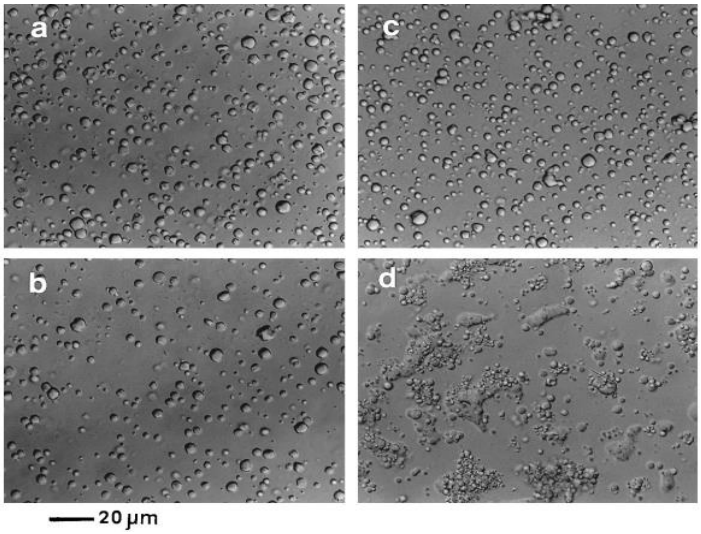


Figure 8 - Photon micrographs of dairy creams. (a,b) Pasteurized and (c,d) sterilized cream. (a,c) Nonpressurized cream controls; (b) cream samples processed at 450 MPa and 10 °C for 30 min; (d) at 450 MPa and 25 °C for 15 min. Adapted from Dumay et al. (1996).

Pasteurized creams (pH 6.7 – 6.8) were subjected to 450 MPa and 25 °C for 15-30 minutes (min) or at 10 °C for 30 min. One day after HPP treatment, the size distribution index was measured, and little variation was displayed, not significantly differing from that of unpressurized cream. In order to evaluate the physical stability of pasteurized cream in storage at 4 °C, FG size distribution was measured over 7 days after HPP. In most cases, no significant differences were noted, and no FG aggregation or coalescence occurred. HPP (450 MPa for 30 min) carried out at 40 °C induced variable effects, depending on the cream batch. Knowing that the caseins adsorbed onto the FGM could be modified by pressure, the FG aggregation was probably due to a decrease in surface charge or an increase in hydrophobicity of the FGM, involving weak Van der Waals interactions. In the case of sterilized cream, because of the effects of

this thermal treatment on the FGM, FG aggregation took place whether the temperature during HPP (450 MPa for 30 min) was 10, 25 or 40 °C, being more sensitive than pasteurized cream to aggregation phenomena. This aggregation could be partly reversed during chilled storage (Dumay *et al.*, 1996).

The pH of creams remained unchanged immediately after HPP, and no acidification took place upon storage for 8 days at 4 °C, in contrast to the apparent fermentation of unpressurized thermally pasteurized cream.

Only a few studies have dealt with the effects of high pressure on food emulsions (Trujillo, 2002). Buchheim and Abou El-Nour (1992) also processed pasteurized dairy creams to between 100 and 500 MPa at 23 °C for 1 to 15 min. Using the freeze- fracture technique and transmission electron microscopy, they found that pressurization induced fat crystallization within the small emulsion droplets, mainly at the globule periphery. Fat crystallization increased with the length of pressure treatment and was maximal after processing at 300-500 MPa. The crystallization proceeded after pressurization during further storage at 23 °C. The authors suggest that extensive fat crystallization induced by HPP has some potential applications such as fast ageing of ice cream and physical ripening of dairy cream for butter making. Furthermore, the whipping properties were improved when cream was treated at pressures up to 600 MPa for up to 2 min, probably due to better crystallization of milk fat. However, if treatment exceeds the optimal conditions, an excessive denaturation of whey protein could occur, resulting in longer whipping time and destabilization of whipped cream (Trujillo, 2002).

For water and non-fatty products, adiabatic heat is approximately 3 °C per 100 MPa. However, fats have larger adiabatic heat, up to 10 °C per 100 MPa, due to higher compressibility of fat compared to water (Trujillo, 2002). High pressure application on fat rich products can induce autoxidation, which can represent a treatment limitation. Butz *et al.* (1999) studied the influence of pressures up to 600 MPa on a model system close to milk fatty acid composition and concluded that, although oleic acid was not affected, pressures from 350 MPa increased linoleic acid autoxidation. However, the effects were small and no new oxidation products were formed, unlike thermal treatment effects.

Many studies have been carried out on the pressure inactivation of pathogenic microorganisms (naturally present in or artificially contaminating) in milk and dairy products. These studies have shown that HPP at a pressure level of 400-600 MPa can enhance the microbiological safety to a similar extent as heat pasteurization conditions (72 °C for 15 s) (Neetoo and Chen, 2012).

Raffalli *et al.* (1994) showed that it is possible to reduce significantly the microbial load of dairy cream (35% fat) by HPP at 450 MPa and 25 °C for 10 to 30 min, with minimal adverse effects on the rheological properties of dairy creams, average size of fat globules, or on the pH value of samples. Consequently, in order to extend the refrigerated storage life of dairy creams, the authors pointed out the possibility of applying HPP.

1.4.3. High pressure inactivation of inoculated microorganisms

In order to study the behaviour of microorganism in foodstuffs, it is necessary to inoculate the organism into the food (Voysey *et al.*, 2009). Gervilla *et al.* (2000) studied the effect of different treatment conditions (pressure and temperature) on the inactivation of five bacterial species: *Escherichia coli*, *Pseudomonas fluorescens*, *L. innocua*, *S. aureus* and *Lactobacillus helveticus* inoculated in ovine milk with different fat content. *E. coli* is considered to be a good index of direct or indirect contamination of fecal origin; *P. fluorescens* is an indicator for *Pseudomonas* spp. which is the major component of the spoilage flora present in refrigerated milk; *L. inoccua* is an indicator for *L. monocytogenes*, a human pathogen and possible contaminant of milk and dairy products; *S. aureus* is a pathogenic that has been involved in numerous food-poisoning outbreaks in milk and dairy products; and *Lb. helveticus*, a microorganism of technological interest in cheese production, which can also be used as a starter culture in the manufacture of some fermented dairy products. These microorganisms were inoculated at a concentration between 10^7 and 10^8 CFU/mL in Ringer solution and ovine milk. Pressurization treatments were carried out at pressures between 100-500 MPa and at temperatures of 4, 25 and 50 °C for 15 min. Each microorganism had a different response to HPP treatments (more destruction *P. fluorescens* > *E. coli* ≥ *L. innocua* > *Lb. helveticus* > *S. aureus*). The authors observed that higher pressure gave higher lethality, for all microorganisms. On the contrary, for some microorganisms, higher temperatures during the pressurization treatments of samples did not increase the lethality.

Another study, carried out by Alpas *et al.* (2000), indicated that HPP in conjunction with mild heat and acidity can be an effective method when it comes to inactivating pressure-resistant and pressure-sensitive strains of four foodborne pathogens (*S. aureus* 485 and 765, *L. monocytogenes* CA and OH_2, *E. coli* O157:H7 933 and 931, *S. enteritidis* positive and Gram-negative strains of pathogens are given in **Tables B2** and **B3 (Appendix – B)**, respectively.

Analysing the four different strains of Gram-negative bacteria at 345 MPa, it is noticeable that their viability loss reached over 8 log cycles, even at 35 °C in 5 min (**Table B3 – Appendix B**). At 50 °C, all of pathogens' strains studied showed more than 8 log cycle reduction within 5 min, except for *S. aureus* 485, that seemed to be the most resistant strain.

Given the data showed on **Tables B2** and **B3** (**Appendix B**), it was possible to conclude that the viability loss of all Gram-positive strains studied, except for *S. aureus* 485 and *L. monocytogenes* CA, reached 8 log cycles reduction when cells were pressurized at 276 MPa at 50 °C, and at 345 MPa at 45 and 50 °C. The Gram-negative strains, only reached the 8 logs cycle reduction at 276 MPa at 45 °C (only for *S. typhimurium*) and 50 °C (for all strains, except for *E. coli* O157:H7 933), and at 345 MPa at 35, 45 and 50 °C. Furthermore, the authors also concluded that in order to achieve a 6 log cycles reduction of the foodborne pathogens vegetative cells at room temperature, it is necessary to use pressures from 600-700 MPa during 15 min or over 40 min at 350 MPa (Alpas *et al.*, 2000).

1.5. Objectives of this work

The main objective of this part of the work relies on the comparison between a dairy cream treated with high pressure and a thermally pasteurized one, aiming to evaluate the effect of HPP on:

- Microbial load (total aerobic psychrophiles, lactic acid bacteria, *Enterobacteriaceae*, *Escherichia coli* and *Listeria innocua*);
- Colour properties;
- Rheological properties
- Fatty acid composition;
- Volatile profile

The results obtained will allow to confirm the possible feasibility of HPP as a safe processing method, with minimal physicochemical changes on dairy creams, and as a possible alternative to thermal treatments.

PART II

CARRIED OUT IN FOGGIA, ITALY

CHAPTER I - LITERATURE REVIEW

THIS SECTION COMPRISES A BRIEFLY COMPILED LITERATURE REVIEW REGARDING EMULSIONS BASED ON EXTRA VIRGIN OLIVE OIL AND ULTRASOUND TECHNOLOGY

1. INTRODUCTION

1.1. Extra virgin olive oil (EVOO): origin and importance

The olive is the fruit (drupe) of the olive tree (*Olea europaea L.*), which belongs to the family Oleaceae, and is considered as one of the most important tree crops in the world, especially in Mediterranean countries, due to its oil production (Owen *et al.*, 2000; Rizwan *et al.*, 2018). The cultivation of the olive tree and the production of olive oil from the mature fruit, are essential parts of farming practices in the Mediterranean basin (Owen *et al.*, 2000). In this area, the high economic importance of olive tree is responsible for olive growing (or olive industry) being one of the most widespread agricultural activities. It is estimated that about 8 million hectares are cultivated worldwide with olive trees (Tarchoune *et al.*, 2019).

Olive oil is a fundamental constituent of the Mediterranean diet, being considered one of the most health-promoting nutritional diets worldwide (Genovese *et al.*, 2015). Its world production is around 2,000,000 tons, contributing with about 4% of total vegetable oil production. Spain, Italy, Greece and Maghrebian countries are the major olive oil producer countries in the world (Visioli *et al.*, 2002). Olive oil is mainly composed of triacylglycerols or triglycerides (TG; ~99%), and secondarily of free fatty acids (FFA), mono- and diacylglycerols, hydrocarbons, sterols, aliphatic alcohols, tocopherols and pigments (Blekas *et al.*, 2006). One of the things that differ olive oil from other vegetable oils, is its abundance of oleic acid (18:1 n-9), a monounsaturated fatty acid (MUFA) ranging from 56 to 84% of total FA. Linoleic acid (18:2 n-6), which is the main essential FA and the most abundant polyunsaturate FA in our diet, is present in concetrations between 3 and 21% (Visioli and Galli, 1998). Olive oil can be classified into different grades, depending on its chemical and organoleptic properties. This classification can also serve as guideline for consumers, who can choose their preferred kind of oil. Extra virgin olive oil (EVOO) accounts for 10% of all the oil produced worldwide, and it is the most valuable kind of olive oil, being obtained from intact olives that are quickly processed and cold-pressed, minimizing cellular lipases activation and consequent degradation of TG (Visioli *et al.*, 2002). Olive oil's importance is related to its high amounts of MUFA and to the presence of low-represented components such as α-tocopherol, phenolics, chlorophyll and carotenoids (Tarchoune *et al.*, 2019). The unique and balaced FA composition of EVOO is responsible for its role in reducing the risk of cardiovascular diseases and also in regulating blood cholesterol levels. In addition, phenols are among

the most important nutraceutical compounds of olive oils, because of their protective characteristics, presenting antioxidant and antimicrobial effect, which contribute in defending against diseases like cancer and atherosclerosis, and also due to their influence on olive oil's quality and organoleptic characteristics (Rizwan *et al.*, 2018; Tarchoune *et al.*, 2019). Particular attention has been paid to phenolic compounds. Several papers correlating the *in vitro* and *in vivo* positive actions of EVOO's chemical compounds on human health have been published (Sacchi *et al.*, 2014).

1.2. EVOO functional compounds

Mediterranean diet has indeed been associated with a reduced incidence of certain pathologies related to chronic inflammations and immune system pathologies. Olive oil, the main source of fat in this type of diet, can also help to reduce inflammation (Rosillo *et al.*, 2018). The EVOO has particularly high standards both in terms of technological parameters related to its oxidative condition and potential shelf-life, namely acidity, peroxide values and oxidation indices, as well as sensory characteristics assessed by recognized panels (Caporaso, 2016). Its health benefits are not only due to its high content in MUFA, but also due to its minor highly bioactive compounds, which includes phenolic compounds such as hydroxytyrosol, tyrosol and oleuropein (Rosillo *et al.*, 2018).

Among the secondary plant metabolites, polyphenols could be considered as the most important group of plant compounds, being chemically characterized by the presence of one or more aromatic rings with one or more hydroxyl substituents. Natural phenolic substances are produced by plants to combat pests and bacterial infections. Flavonols, lignans and glycosides are among the polyphenols produced by the olive tree (*Olea europaea*). In EVOO, the amount of phenolic compounds depends on several factors, including (1) the olive cultivar and the ripening stage of the fruit; (2) environmental factors; (3) extraction conditions (heating, added water and malaxation); (4) extraction systems used to separate oil from olive pastes (pressure, centrifugation systems); and (5) storage conditions and time, due to spontaneous oxidation and suspended particle deposition (Rigacci and Stefani, 2016). Usually, polyphenol content decreases with over-maturation of olives, however, if the olives are grown in warmer climates, regardless of more rapid maturation, it will produce oils that are richer in phenols. On the other hand, olives that are hand-picked at the right moment, immeadiately taken to the mill and processed at temperatures lower than 25-30 °C, also produce high quality oils with high phenolic content (Visioli *et al.*, 2002). Simple phenols (hydroxytyrosol, tyrosol),

secoiridoids (oleuropein), and lignans, are the three different classes where major phenolic compounds were identified and quantified in olive oil (Owen *et al.*, 2000). Olive oil polyphenols are a complex mixture of compounds with different chemical structures, which are normally related to the oil oxidative stability and shelf-life, and also to its sensory aspects and biological properties (Giacintucci *et al.*, 2016).

A daily consumption of olive oil containing at least 5 mg of hydroxytyrosol and its derivatives per 23 g of olive oil, was associated with the protection of blood lipids from oxidative stress, being recognized by the European Food Safety Authority (EFSA) in 2011. Among the most characteristic hydroxytyrosol derivatives are, tyrosol, oleocanthal, oleacein, oleuropein and ligstroside aglycons (Nikou *et al.*, 2019). Most phenols confer a very bitter and pungent taste to the oil, thus, oils that lack phenols, have a more "sweet" taste. Furthermore, it is also important to note that very high loads of phenols, can originate a very high level of bitterness and pungency which can be unpleasant for some people, however, this is not synonymous of lower quality (Visioli and Galli, 1998). The secoiridoid oleuropein is responsible for the bitter taste of olives, and it is found, together with its aglycon form, in olive oil. During maturation, oleuropein undergoes hydrolysis, yielding several simpler molecules which build up the well-known olive oil complex taste (Visioli *et al.*, 2002).

Oxidation is the main cause of quality deterioration of olive oil, due to its high concentration of unsaturated fatty acids. EVOOs are known to be more resistant to oxidation than other oils, due to their lower unsaturation and their unsaponificable components, such as tocopherols and phenolic compounds (Di Mattia *et al.*, 2009). Given their antioxidative properties, phenolic compounds play an important role against oxidative stress, being able to extend EVOO shelf-life (Tarchoune *et al.*, 2019). In order to determine the efficiency of an antioxidant to protect lipid auto-oxidation in an emulsion system, its necessary to take some parameters into account such as its polarity, its activity in terms of capacity and rapidity to donate an hydrogen atom, and its localization among the three phases, lipid, water and interface (Di Mattia *et al.*, 2009).

Oxidative modifications of phenolic compounds can also be advantageous sometimes if they result in enhanced aroma and flavour of food, in this case olive oils (Visioli *et al.*, 2002). During EVOO cooking, changes in phenolic and volatile compounds can occur. Using raw EVOO on food, for instance as a salad oil, is the best way to obtain its original flavour while also maximizing the intake of its natural antioxidants and compounds associated with positive effects on human health. However, even during cooking, EVOO

exhibits strong antioxidant properties and influences the overall flavour of cooked foods. It can induce changes in the sensorial (bitterness and fruity flavour) and nutritional qualities. Furthermore, interactions between phenolic compounds of EVOO and other compounds present in different cooked food systems, that might occur during cooking, can influence the healthy and protective effect of these cooked foods (Sacchi *et al.*, 2014).

The technological properties of EVOO in emulsified food systems and the role of its complex composition (e.g. FFA, phospholipids and polyphenols) on the physical properties of the systems and their stability has been investigated (Di Mattia *et al.*, 2010, 2015). The results of these studies, have shown that some surface active endogenous amphiphylic molecules may affect the formation of oil/water interfaces and the physical and chemical stability of dispersed emulsified systems (Di Mattia *et al.*, 2015).

Di Mattia *et al.* (2015) studied the physical and structural properties of mayonnaise made with different EVOOs, selected according to their polyphenolic content, and noticed that the dispersion degree and physical properties of the mayonnaise samples were deeply affected by the concentration of polyphenols of the oils. They observed that the droplet size of the EVOO based mayonnaise would increase with increasing phenolic content of the oil. Furthermore, they observed a decrease of firmness and consistency in products obtained with EVOOs containing lower phenolic concentrations. Later, Giacintucci *et al.* (2016) also studied mayonnaise systems using two different EVOOs, one with high polyphenolic content (HP-EVOO), in particular oleuropein (90% of total compounds), and another with lower content of phenolics and oleuropein (LP-EVOO), characterized by a much wider phenolic pattern (tyrosol, caffeic acid, p- and o-coumaric and ferulic acids). Mayonnaise made with HP-EVOO were characterized by a high degree of polidispersity (**Figure 9**) and larger oil droplets, with a broader and coarser distribution of fat globules. In contrast, LP-EVOO-made mayonnaises showed a lower degree of fat globule polidispersity (**Figure 9**), which could be attributed to the lower phenolic content and, in particular, of oleuropein. The authors also analysed the samples after two weeks of storage, and noticed that the HP-EVOO-made mayonnaise showed major changes, with a broader droplet size distribution and a decrease of percentage of smaller particles. Furthermore, these mayonnaises presented a tendency to oiling off during storage, which limited their further storage.

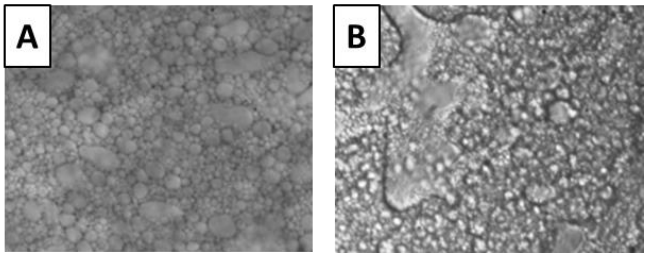


Figure 9 – Optical micrographs of the mayonnaise produced using LP-EVOO (**A**) and HP-EVOO (**B**). Adapted from Giacintucci *et al.* (2016).

In order to understand the specific technologcal functionality of olive polyphenols, as well as of the other amphyphylic EVOO compounds in complex emulsified structures, further investigations are needed.

1.3. Emulsions

Many natural and processed foods such as mayonnaise, salad dressings, cream liqueurs and butter, for instance, are examples of partial or whole emulsions, and that also have a role as ingredients in the formation of more complex products such as yoghurts, ice creams and whipped products (Di Mattia *et al.*, 2011; Caporaso, 2016).

Emulsions are thermodynamically unstable colloidal dispersions that consist of a mixture of two immiscible liquids, in which one is dispersed as fine droplets (dispersed phase) into the other liquid (continuous phase). They can be classified based on droplet size as conventional emulsion, microemulsion and nanoemulsion, and based on dispersed phase properties, as oil-in-water (O/W) or water-in-oil (W/O) depending on whether the continuous phase is water or oil, respectively (**Figure 10**) (Lu and Gao, 2009; Kumar and Sarkar, 2017).

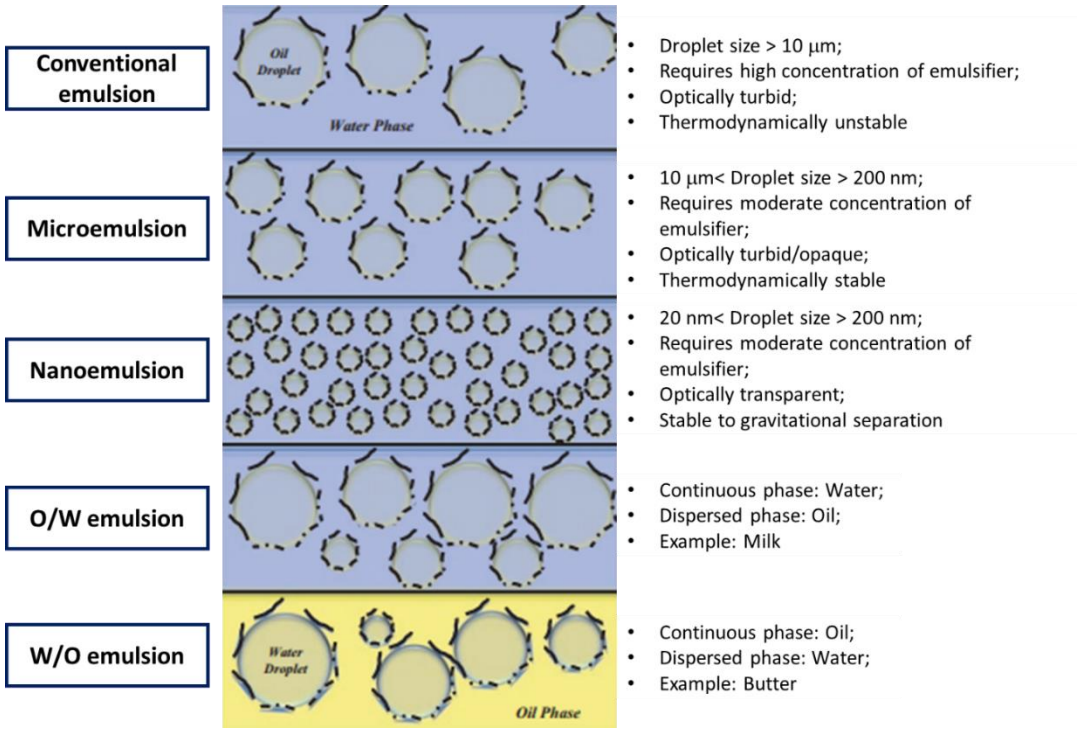


Figure 10 - Characteristics of different emulsions, based on their droplet size and dispersion phase properties. Adapted from Kumar and Sarkar (2017).

The two phases will eventually separate if they are allowed to stand for enough time, thus emulsions' stability can be defined as their ability to resist changes in its physicochemical properties over time, therefore, processes such as flocculation, creaming/sedimentation, coalescence, phase inversion and Ostwald ripening contribute to their physical instability, which in turn decreases the product quality and shorten shelf-life (Caporaso, 2016; Hu *et al.*, 2017) (**Figure 11**).

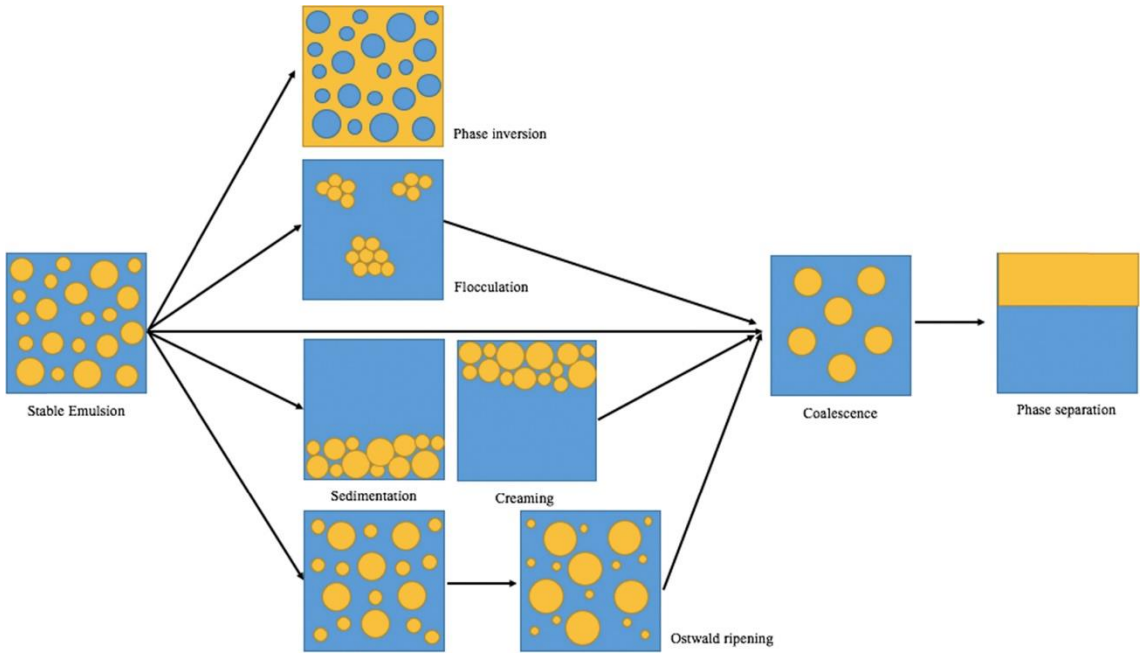


Figure 11 - Schematic representation of physical instability mechanisms in emulsions. Adapted from Hu *et al*. (2017).

Furthermore, emulsions' compositional materials, processing conditions and interfacial properties (e.g. tension, rheology, charge and contact angle) highly influences their stability and functionality (Hu *et al.*, 2017; Kumar and Sarkar, 2017). Moreover, the stability of emulsions is dependent upon the characteristics of emulsion droplets, such as radius and density, and of continuous phase, such as viscosity and density (Caporaso, 2016). Most of the time, emulsion instability can be observed by the naked human eye, which is the case of gravity separation, such as creaming and sedimentation. However, for observing phenomena like flocculation, coalescence and Ostwald ripening, analytical instruments such as microscope are needed (Hu *et al.*, 2017).

In order to produce an emulsion, it is necessary to apply enough energy to the oil/water interface, thus breaking up the bulk oil into smaller droplets (Hu *et al.*, 2017). Besides breaking up the droplets, it is necessary to stabilize the freshly generated interface, with an emulsifier, to prevent recoalescence of the newly formed droplets. Thus, these emulsions can be stabilized by an emulsifying agent (surfactant) (**Table 5**), which are substances that can reduce the surface tension at the interface of the immiscible phases, allowing them to mix and form an emulsion, due to the fact that less external pressure is required for droplet disruption (Kinyanjui *et al.*, 2003; Lu and Gao, 2009; Abbas *et al.*, 2013). After the disruption process and the formation of new droplets, emulsifiers adsorb to the oil-water interface, forming a protective coating around the newly formed droplets, which helps prevent droplet aggregation during and after

emulsion formation (Komaiko, 2016). The formation of O/W or W/O emulsions depends on the surfactant solubility, i.e., if it is more soluble in water (hydrophilic surfactants) or oil (lipophilic surfactants), respectively (Lu and Gao, 2009). Surfactants such as monoglycerides and phospholipids are commonly used in foods to stabilise interfaces, due to their amphiphilic nature (Caporaso, 2016). On the other hand, proteins and polysaccharides are important biopolymers used in food emulsions to control their texture, microstructure and also stability. While polysaccharides are employed as thickeners, enhancing viscosity or forming a gel, proteins can form networks and also have emulsification and foaming properties (Herrera, 2012; Caporaso, 2016). Despite being necessary, emulsifiers in the food industry are desirable in minimum amounts to form stable nanoemulsions, due to practical, health and economic reasons (Katsouli *et al.*, 2017).

Table 5 - Types of surfactants used in food grade emulsion formation. Adapted from Komaiko (2016).

Surfactant type	Example/Source
Small molecule surfactants	Tweens, Spans
Phospholipids	Egg, soy, sunflower, or dairy lecithin
Amphiphilic proteins	Whey protein isolate, caseinate
Amphiphilic polysaccharides	Gum Arabic, modified starches

Despite the fact that nanoemulsions are not thermodynamically stable, when compared to the other types of emulsion shown in **Figure 10**, they present good long-term physical stability, due to their small particle size, which largely prevents or decelerate conventional destabilisation phenomena such as creaming and coalescence. Thus, the main source of instability generally reported is Ostwald ripening (**Figure 11**), which is the tendency of small droplets to merge with larger droplets due to differences in solubility, which in turn, increases the average size of emulsion droplets (Klang *et al.*, 2012; Abbas *et al.*, 2013).

1.3.1. Ultrasound technology

Low production cost, emulsions with smaller particle or droplet size and narrow size distribution, more stable emulsions, less surfactant required for the given droplet size, small footprint, little technical support requirements, ease of system manipulation, high energy efficiency, low instrumental requirements and self-sterilizing potential due to its antibacterial properties, are the main reasons why ultrasonic homogenization has gained so much attention lately (Abbas *et al.*, 2013; Peshkovsky *et al.*, 2013; Mehmood *et al.*,

2017). Based on its frequency range, ultrasound can be classified in two distinct categories, high frequency (100 kHz-1MHz) low power (<1 W cm^{-2}) ultrasound, which is mostly utilized for the analytical evaluation of the physicochemical properties of food, and low frequency (20-100 kHz) high power (10-1000 W cm^{-2}) ultrasound, employed for the alteration of foods, either physically or chemically (Sullivan *et al.*, 2016). Depending on the viscosity and conductivity of the medium, sound waves can dissipate part of the acoustic energy into heat energy while passing through the medium (Abbas *et al.*, 2013).

These devices consist of an ultrasonic probe (**Figure 12**) that contains piezoelectric quartz crystals, which convert inputted electrical waves into intense pressure waves, expanding and contracting in response to an alternating electrical voltage (McClements, 2011; McClements and Rao, 2011).

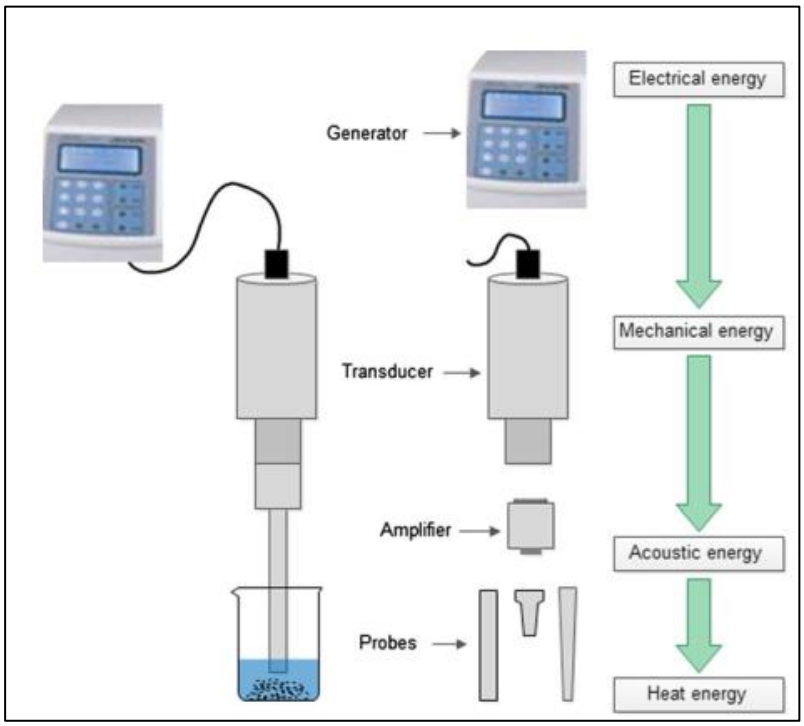


Figure 12 - Schematic representation of an ultrasonic setup, its components, (the generator, transducer, amplifiers and probe-types), and the sequence of energy transformations at different levels of the operation. Adapted from Abbas *et al.* (2013).

Ultrasonic emulsification is believed to occur through two mechanisms. Firstly, when the probe is immersed, ultrasonic waves are transferred to the sample, exerting a pressure known as acoustic pressure, which is dependent on time, frequency and the maximum pressure amplitude of the wave (Kentish *et al.*, 2008; Abbas *et al.*, 2013). The generated waves travel through the liquid, causing microturbulences and an interfacial movement which makes the boundary phase unstable. Thus, the dispersed (internal) phase eventually

breaks, forming droplets in the continuous (external) phase. Secondly, the application of low frequency, high power ultrasounds generates acoustic cavitation (predominant phenomena at high amplitudes), i.e., pre-existing micro-bubbles of gas dissolved into the liquid medium grow in size, due to rectified diffusion, until they become unstable and violently collapse (**Figure 13**). Each bubble collapse (an implosion on a microscopic scale) event causes locally extreme conditions such as very high shear, liquid jets, and extreme heating and cooling rates. These extreme forces break primary droplets of dispersed phase into nanosized droplet and mix them homogeneously into the continuous phase (Suslick, 1995; Kentish *et al.*, 2008; Abbas *et al.*, 2013).

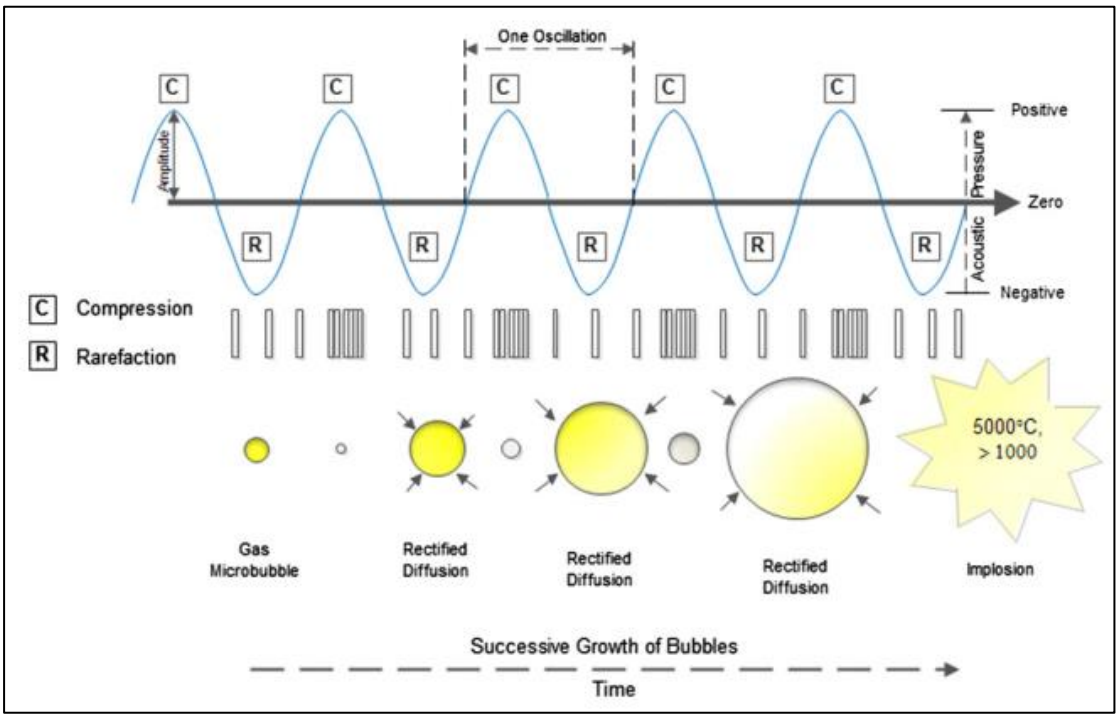


Figure 13 - Schematic diagram of the growth and collapse of a bubble in acoustic cavitation process. Adapted from Abbas *et al*. (2013).

Raviadaran, Ng, Manickam, and Chandran (2019) compared the effect of ultrasound and microfluidizer on some parameters for the formation of a palm oil-based W/O nanoemulsion. Concerning the ultrasound, the authors observed that the mean droplet diameter (MDD), decreased progressively when acoustic amplitude and irradiation time were increased, due to the fact that more acoustic bubbles could be generated and their subsequent implosion intensified the degree of cavitation activity (Raviadaran *et al.*, 2019). As for the microfluidizer, MDD decreases progressively with an increase in the operating pressure and number of cycles, as expected. Furthermore, they also observed that, regardless of the method used, supplying more energy beyond an optimum would not lower the MDD, but instead, would lead to an increase in MDD. At higher

pressures/amplitude and at longer emulsification times, the droplets are energized and the system may become unstable, thus a recoalescence of the droplets may occur, resulting into an increase in MDD due to insufficient concentration of emulsifier (Raviadaran *et al.*, 2019). In terms of energy consumption, Raviadaran *et al*. (2019) reported that the ultrasound was more energy-efficient than the microfluidizer, requiring 9-fold less energy to obtain a W/O nanoemulsion with an MDD of ~220 nm.

The key to efficiently use ultrasound to obtain emulsions is to optimize its parameters (e.g., frequency, intensity, and acoustic power), i.e., determine an optimum ultrasonic energy intensity input for these systems, since an excessive energy input may lead to an increase in droplet size (Kentish *et al.*, 2008; Cabrera-Trujillo *et al.*, 2016). Furthermore, due to the exothermic nature of the sonication process, depending on the period and intensity of sonication, the temperature of the system may rise, affecting negatively some parameters necessary for an optimum emulsion formation. Thus, temperature control is considered one of the most important parameters in high-energy emulsification processes (Hashtjin and Abbasi, 2015).

Besides physical stability, emulsions also need to be stable microbiologically. Heat pasteurization and sterilization are effective treatments to preserve food products that are based on emulsions with respective to microbial safety. Even though these heat treatments can extend these products shelf-life, they also negatively affect emulsion stability and thus, product's quality. Heat treatment may result in flocculation of the oil droplets in the emulsions, resulting in phase separation (Ven *et al.*, 2007). Therefore, thermal treatments may not be the most adequate processing technologies since they rely on extensive heat transfer, which results in significant loss of colour and flavour components. Thus, extensive research has been going on to develop nonthermal technologies for heat sensitive food products. For such products, HPP may be considered as an alternative processing method (Sethi *et al.*, 2017). The next section will be focused on the effect of HPP on emulsion-based food products.

1.4. High pressure processing of food-grade emulsion systems

HPP has demonstrated promising results when it comes to increasing the shelf-life of salad dressings, sauces and other emulsion-based food products without using chemical preservatives and, at the same time, maintaining its sensory and textural quality (Sethi *et al.*, 2017). Sethi, Chauhan and Anurag (2017) studied the effect of high pressure on green mango blended mayonnaise and observed that samples treated at 400 MPa showed a fine,

homogeneous, compact and well dispersed oil-in-water emulsion with small and even size of fat globules (**Figure 14**). On the contrary, samples treated at 600 MPa, showed a coarse, less compact and loose structure with large sized and uneven fat globules.

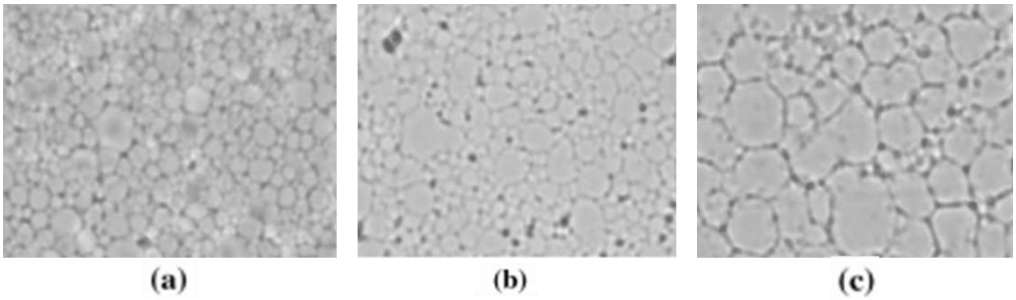


Figure 14 - Microstructural analysis of green mango blended mayonnaise **a)** control (untreated), **b)** treated at 400 MPa and **c)** at 600 MPa. Adapted from Sethi, Chauhan and Anurag (2017).

Furthermore, the authors evaluated the oxidative stability of the mayonnaise in terms of free fatty acids, peroxide value and *p*-anisidine value, and concluded that all these values increased with an increase in pressure level and holding time, but were still within the acceptable limits. Dumay *et al.* (1996) studied the effects of HPP on model liquid oil/water emulsions and did not observe significant changes in droplet size distribution and viscosity of HPP-treated (450 MPa at 10, 25 or 40 °C for 30 min) emulsions prepared with sodium caseinate and peanut oil at pH 7.0.

Some studies have shown that HPP up to 500 MPa for 1 min at 25 °C can eliminate spoilage microorganisms from commercial salad dressings, a high-acid food product, without significantly changing their rheological properties. Arora, Chism and Shellhammer (2003) studied the effect of HPP (800 MPa at 30 °C for 5 min) on acidified model oil-in-water emulsions (pH 3.6) and confirmed that HPP has no significant detrimental effects on the rheological behaviour, as well as physical stability of acidified emulsions stabilized by whey protein isolate and polysorbate-60.

When it comes to antimicrobial activity, HPP (200-500 MPa at 10 °C for 10 min) was able to decrease (>4.0 $\log_{10}$ cycle) the total microbial count of yeasts, moulds and bacteria in stored oil-in-water emulsions prepared with hen egg yolk (Anton *et al.*, 2001). Furthermore, the microbial destruction of HPP treatment is highly influenced by the pH of the medium, thus, in acidic environment, the pressure-sensitivity of microorganisms is much higher than in a neutral medium (Anton *et al.*, 2001).

1.5. Objectives of this part of the work

The main objective of the second part of this work is to obtain a stable emulsion made of water and different cultivars of EVOO, with different total polyphenol content, by combining emulsifiers with ultrasound technology. After optimizing the ultrasound processing conditions and defining the suitable type and amount of emulsifier, the following analyses will be carried out on the obtained emulsions:

- Chemical characterization of each EVOO;
- Physicochemical parameters;
- Rheological properties;
- Accelerated emulsion stability;
- Microstructure and surface morphology observations;

Furthermore, total phenolic content and microbial counts of each emulsion were determined before, immediately after HPP and during storage, to evaluate the potential of this technology on inhibiting microbial growth over storage at room temperature, while keeping the EVOO based emulsions stable.

PART I

CARRIED OUT IN AVEIRO, PORTUGAL

CHAPTER II – MATERIALS AND METHODS

THIS SECTION COMPRISES A DETAILED DESCRIPTION OF ALL THE METHODOLOGIES EMPLOYED IN THE WORK DONE IN AVEIRO

1.1. Cream samples

Industrially homogenized raw (for further HPP) and thermally pasteurized cream from the same batch were kindly provided, twice (different batches), by a local company (Portugal). Both batches were necessary to perform two sets of experiments, in order to test different HPP conditions (time/pressure) and its effect on different microorganisms.

1.2. Preparation of cream samples and inoculation

In order to study the influence of HPP on cream physicochemical properties and microbiology, for each day of storage, 20 mL of each sample, in triplicate, were aseptically packed, in UV-light sterilized, low permeability polyamide-polyethylene (PA/PE) bags (90 micron, Plásticos Macar – Indústria de Plástico Lda., Palmeira, Portugal), and manually heat-sealed, excluding as much air as possible.

1.3. High pressure treatment of samples

HPP treatments were carried out in a pilot scale high pressure equipment, with 55 L of volume and 200 mm of diameter vessel (Hiperbaric Model 55, Burgos, Spain). Before processing, samples were double packaged in a 150 microns PA/PE bag with cold (4 °C) water between the two polymer layers, thus minimizing samples from getting warm during processing due to fat adiabatic heat, which could induce autoxidation (Butz *et al*., Trujillo, 2002). The first cream batch was subjected to 450 MPa and 600 MPa for 5 min each, at 7 °C, and the second cream batch was processed at 600 MPa for 5 and 15 min, also at 7 °C.

After the respective processing, samples from both batches were stored at 4 °C.

1.4. Storage conditions

After processing, thermally pasteurized and HPP cream samples from the first batch were stored under refrigeration temperature (4 °C) for 0, 5, 9, 18, 33 and 51 days, while samples from the second batch were stored for 0, 3, 10, 28 and 52 days, to evaluate and compare the shelf-life of creams processed at both conditions (thermal and HPP pasteurizations) (**Table 6**). Day 0 was set as first day immediately after high pressure/pasteurization treatment.

Table 6 – Experimental design of each cream batch and the aim of study.

	HPP Conditions				
	Pressure (MPa)	**Duration (min)**	**Storage period (days)**	**Nomenclature**	To study the effect of HPP after process and during each storage period on:
1st batch	-	-	0, 5, 9, 18, 33, 51	Raw	• General microbiology and physicochemical parameters
	-	-		Pasteurized	
	450	5		450/5	
	600	5		600/5	
2nd batch	-	-	0, 3, 10, 28, 52	Raw	• General microbiology and physicochemical parameters; • Inoculated *E. coli* and *L. innocua*
	-	-		Pasteurized	
	600	5		600/5	
	600	15		600/15	

1.5. Microbial analyses

After each experiment, cream samples from the first batch were analysed for counts of total aerobic psychrophiles (TAP), *Enterobacteriaceae* (ENT) and lactic acid bacteria (LAB). Apart from ENT, samples from the second batch were analysed for the same microorganisms and also for inoculated *E. coli* and *L. innocua*. 1.0 mL of each cream sample was transferred to tubes containing 9 mL of Ringer's solution under aseptic conditions and homogenized. Further, decimal dilutions were made with the same solution and plated in triplicate on the appropriate media.

1.5.1. Inoculum preparation

Cultures of *E. coli* (ATCC 25922) and *L. innocua* (ATCC 33090) were used to inoculate cream samples from the second set of experiments. Both cultures were stored on Trypticase Soy Agar (TSA; Liofilchem) Petri dishes at 4 ºC. One colony of each microorganism, previously isolated in TSA plate, was collected, inoculated in 250 mL of Tryptic Soy Broth (TSB; Liofilchem, Italy) in a 500 mL Erlenmeyer flask (to allow proper agitation) and incubated at 37 ºC, 150 rpm, during 10-12h. Then, 1 mL of the previous culture was transferred to 250 mL of fresh TSB and left again overnight in the same temperature and agitation conditions. The growth curve of each microorganism had already been previously obtained by this research group in order to estimate the several growth phases over time. Thus, before inoculation, the concentration of each microorganism was confirmed through the optical density of the culture, estimated on this

previously obtained growth curve. This growth period was selected in order to ensure that cells reached the stationary phase to be later inoculated into raw cream, with a final concentration of about 10^8 cells/mL. Under aseptic conditions, twenty millilitres of each cell suspension were used to inoculate 160 mL of the second batch of cream samples.

It is well documented in the literature that bacterial cells in the stationary phase exhibit greater pressure tolerance than exponentially-growing cells (Clements *et al.*, 2001; Mackey *et al.*, 2009; Balasubramaniam *et al.*, 2016). Therefore, in this study, bacteria were inoculated at stationary phase to simulate the worst-case scenario. Sometimes HPP can result in sub-lethally injured cells which cannot be detected on selective media, and thus, these cells can potentially repair themselves and cause disease (Stratakos *et al.*, 2019).

1.5.2. Total aerobic psychrophiles counts

Total aerobic psychrophiles counts were enumerated using 1.0 mL of diluted solution sample, on plate count agar (PCA, Merck), by incubation at 20 °C for 5 days (ISO 4833:2003) (International Organization for Standardization, 2003).

1.5.3. *Enterobacteriaceae* counts

Enterobacteriaceae were quantified in violet red bile dextrose agar (VRBDA, Merck), by pour-plated method using 1.0 mL of diluted solution sample, being incubated at 37 °C for 24h (ISO 8523:1991) (International Organization for Standardization, 1991).

1.5.4. Lactic acid bacteria counts

Lactic acid bacteria counts were enumerated on Man Rogosa and Sharpe agar (MRS, Merck) medium, by pour-plated method using 1.0 mL of diluted solution samples, being incubated at 30 °C for 5 days (ISO 15214:1998) (International Organization for Standardization, 1998).

1.5.5. *Escherichia coli* counts

Escherichia coli counts were determined on chromogenic coliform agar (CCA, Merck), by pour-plated method using 1.0 mL of diluted solution samples, being incubated at 37 °C for 24 h (ISO 7251:2005) (International Organization for Standardization, 2005).

1.5.6. *Listeria innocua* counts

The viable counts of *Listeria innocua* were determined on plates with PALCAM agar base added with *Listeria* selective supplement (FD061) (Liofilchem), by pour-plated method using 1.0 mL of diluted solution samples. The plates were incubated at 37 °C for 48 h (ISO 11290-1:1998) (International Organization for Standardization, 1998), being the green colonies surrounded by a black zone counted.

1.6. Microbial counts

In all cases, Petri dishes containing 15-300 colonies forming units (CFU) were selected for counting. The obtained results were expressed as decimal logarithm of colony forming units per millilitre of cream (Log_{10} CFU/mL). The maximum endogenous microbial load considered in this study was 6.00 log CFU/mL (Institute of Medicine (US) and National Research Council (US) Committee, 2003), while the detection limit for this method was 1.00 log CFU/mL.

Microbial counts were calculated using the following equation (1):

$$\boldsymbol{Log_{10}(N) = \frac{\sum Characteristic\ colonies}{V\,[(n_1+0.1\times n_2)\times d]}} \qquad \textbf{(Equation 1)}$$

Being:

$\boldsymbol{N}$ – Colony forming units per mL of samples (CFU/mL)

$\boldsymbol{V}$ – Sample volume (mL)

$\boldsymbol{n_1}$ – Number of plates in the 1st dilution

$\boldsymbol{n_2}$ – Number of plates in the 2nd dilution

$\boldsymbol{d}$ – 1st dilution

1.7. pH analysis

The pH of all cream samples was measured at room temperature (21 ± 2 ºC) with a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S.A., Spain), with temperature compensation. For each sample, the pH was measured in triplicate.

1.8. Colour measurement

Colour measurements were performed using a spectrophotometer Konica Minolta CM 2300d (Osaka, Japan). This spectrophotometer was calibrated before each colour measurement. Furthermore, samples were gently mixed before each experiment. Measurements were done by selecting three random spots in each sample. Colour parameters were recorded according to the *Commission internationale de l'éclairage*

(CIE) system and the data was processed with the original SpectraMagic™ NX software (Konica Minolta, Osaka, Japan) in accordance to the International Commission on Illumination regulations: L^* - lightness (0, dark; 100, light), a^* - redness (+, red; –, green) and b^* - yellowness (+, yellow; –, blue). These colour parameters were measured and the total colour change variation (ΔE^*) was calculated by the following equation (2):

$$\Delta E^* = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2\,]^{1/2} \qquad \textbf{(Equation 2)}$$

where ΔE^* is the total colour change between a sample and the control (initial values identified with the subscript "0"). By following the same method as Stratakos *et al.* (2019), depending on ΔE^* value, the colour difference between treated and untreated samples was estimated such as not noticeable (0-0.5), slightly noticeable (0.5-1.5), noticeable (1.5-3.0), well visible (3.0-6.0) and great (6.0-12.0).

1.9. Rheological properties

Cream rheological characteristics were determined using a controlled-stress rheometer (AR-1000, TA Instruments, New Castle, DE) equipped with a cone-and-plate geometry (acrylic cone, 6 cm diameter and 2° angle). The temperature of the bottom plate was controlled by a circulating bath (Circulating Bath 1156D, VWR International, Carnaxide, Portugal), to ensure constant temperature during the test. Samples were gently homogenized and placed carefully (approximately 2 mL) on the top of the bottom plate to minimize damage of the sample structure and to avoid trapping of air bubbles. Prior to all tests, samples temperature was equilibrated to 25 °C for about 15 min. Flow curves were obtained by applying a continuous stress ramp (0 to 3 Pa) for 3 minutes.

1.10. Fatty acid determination

Fatty acid (FA) content was determined by gas chromatography, as fatty acid methyl esters (FAMEs). In brief, fat was separated by centrifugation at 13000 rpm for 20 min. A 40 µL portion of the upper layer (fat phase) was dissolved in hexane (2 mL) and the fatty acids were converted to their respective FAME by cold transmethylation (ISO 12966-2:2011, 2011). Chromatographic separation was achieved on Agilent JandW Select FAME column (100 m × 0.25 mm, JW) using a Chrompack CP 9001 gas chromatograph (Chrompack, Middelburg, The Netherlands) equipped with a FID detector. Fatty acids identification and FID calibration was accomplished with a certified reference mixture of

fatty acids methyl esters (TraceCert – Supelco 37 component FAME mix, Supelco). Fatty acids were expressed relative percentage of their FAME.

1.11. Volatile profile

Volatile compounds (VOCs) profile was determined by headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS), according to the methodology applied by Shepard *et al.* (2013), with some modifications. Initially 5 mL of each sample were placed in 20 mL headspace vials, then cyclohexanone was added as internal standard (5 µg from an aqueous solution) along with 28% sodium chloride (w/w) and a magnetic stir bar, being the vials immediately sealed with a polypropylene cap with silicon septum. The vials were heated at 60 °C for 20 min with constant stirring (250 rpm). After that, the SPME fiber coated with divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm; Supelco Inc.) was exposed in each sample during 30 min, still at 60 °C, for volatiles adsorption. Volatiles were thermally desorbed for 5 min in the injector port (splitless mode; 250 °C) of the GC-EI-MS system. Chromatographic separation was performed on a fused-silica DB-5 MS Capillary GC column (30 m ×0.25 mm I.D. × 0.25 µm film thickness, Agilent) with a temperature program from 40 °C to 235 °C, with a total run time of 60 min. The MS transfer line and ion source were at 280°C and 230ºC respectively, and MS quadrupole temperature at 150 °C, with electron ionization of 70 eV; set in full scan mode (m/z 40 to 650 at 1.2 scan/s). Compounds were identified by comparing the respective mass spectra with a mass spectral database (NIST v14, nist.gov), and semi-quantification achieved as internal standard equivalents basis, express in µg of internal standard equivalents per 100 ml of cream.

1.12. Statistical analysis

The experiments were carried out in triplicate and all analysis were done in duplicate. Statistical data analysis of the results was performed using the Analysis of variance (ANOVA), followed by a multiple comparison post-hoc test, Turkey's honest significant differences (HSD) test, at a 5% level of significance.

PART II

CARRIED OUT IN FOGGIA, ITALY

CHAPTER II – MATERIALS AND METHODS

THIS SECTION COMPRISES A DETAILED DESCRIPTION OF ALL THE METHODOLOGIES EMPLOYED ON THIS PART OF THE WORK

1.1. Materials

Four extra virgin olive oils (EVOO) from different olive cultivars (Coratina, Peranzana, Arbequina and A07) were kindly offered by Azienda Agricola Salvatore Moffa (Torremaggiore, Foggia, Italy). These four cultivars were selected due to their different content in polyphenols. A sunflower oil of a commercial brand was used as a control treatment, since it has zero polyphenol content.

Three emulsifiers, that are most used in the food industry, were tested in order to obtain a stable emulsion with EVOO. Soy lecithin (E322), purchased from Foxwood Industrial Park (Chesterfield, UK), mono- and diglycerides of fatty acid (MDG) (E471), purchased from Laboratori Bio Line S.r.l (Canaro, Italy) and sucrose ester of fatty acids (E473), purchased from Barticular S.r.l. (Perugia, Italy).

The mineral water used to prepare the emulsions was bought in local markets (Foggia, Italy). Its chemical characteristics are reported in **Table C1** (**Appendix – C**).

1.2. Emulsion production by ultrasound treatment

The emulsions were prepared using an ultrasonic device Hielscher model UP200St-T (Seneco S.r.l., Teltow, Germany) (**Figure D1 – Appendix D**) capable of producing sub-micron and nano stable food emulsions and equipped with an ultrasonic transducer, an ultrasonic generator (200 W, 26 KHz), automatic frequency regulation, amplitude adjustment from 20 to 100%, impulse adjustment from 0 to 100% and a temperature sensor for monitoring (range -50 to 200 °C).

All parameters were monitored during treatment by connection of the ultrasound device to computer.

The emulsions were prepared by immersing the probe in the centre of the of the glass beaker at a depth of ~ 1 cm and the temperature sensor was placed at 1 cm distance from ultrasound probe to avoid interferences. The glass beaker was covered by ice to prevent overheating (< 25 °C) and the temperature was monitored during each sonication treatment.

1.3. Factorial plan to evaluate the effects of ultrasound parameters on emulsion stability

The effect of different parameters of ultrasound treatment on the stability of emulsions made with EVOO were evaluated by a factorial plan. In these experiments no emulsifiers were added. The ultrasound treatments were carried out at five percentages of amplitude

and power (20; 40; 60; 80 and 100%) and pulse cycle (10; 20; 30; 40 and 50%). Coded and actual values of variables are shown in **Table D1** (**Appendix D**). The factorial design of these three variables (amplitude, power and pulse cycle) and five levels of values were elaborated according to Central Composite Design (CCD) (Box *et al.*, 1978). This method was used to evaluate the single influences of the processing variables as well as their possible interactions. Seventeen tests with different combinations of process variable values were obtained (**Table 7**).

Table 7 - Experimental factorial plan elaborated to evaluate the effects of ultrasound parameters on stability of emulsion made up of EVOO. Values with a * represent the central point.

Samples	Amplitude (%)	Power (%)	Pulse cycle (%)
1	40	40	20
2	40	40	40
3	40	80	20
4	40	80	40
5	80	40	20
6	80	40	40
7	80	80	20
8	80	80	40
9	26.36	60	30
10	93.64	60	30
11	60	26.36	30
12	60	93.64	30
13	60	60	13.18
14	60	60	46.82
15	60*	60*	30*
16	60	60	30
17	60	60	30

1.4. Emulsion preparation by addition of different emulsifiers

The initial percentage of all emulsifiers used were based on the ones advised on the respective emulsifier label. After that, in order to decrease these percentages and, also avoid phase separation, some preliminary tests were performed. In the end, the most suitable emulsifier was chosen.

Samples with added soy lecithin were prepared by firstly adding 0.9% of the emulsifier in 55.1% of water at room temperature, and then adding 4% of lemon juice to the mixture, in order to reduce the pH and help prevent possible microbial growth

(Kadhim Hindi and Ghani Chabuck, 2013). Lastly, after mixing with a spoon, 40% of EVOO was added and the obtained mixture was submitted to ultrasound treatment.

When concerning MDG emulsifier, samples were prepared in a two-stage process. The amount of emulsifier tested in the preliminary test is related to the water phase in the following ratios: 1:100; 1:50; 1:40; 1:25; 1:10; 1:6; 1:5 and 1:4.

At first, these different amounts of emulsifier were dissolved in hot mineral water (80 °C). After complete dissolution of the emulsifier, 2% of lemon juice was added. Finally, 40% of EVOO was added to this mixture and submitted to sonication.

On samples prepared with sucrose ester of fatty acids, this emulsifier was added at a percentage of 1% to mineral water (room temperature), as indicated in the label. Also, in this case, 2% of lemon juice was added and the percentage of EVOO was 40%. After the preparation of this mixture, the ultrasound treatment was applied.

The values of ultrasound parameters used to produce samples were chosen as a function of results obtained from factorial plan reported in ***"section 1.3."***

1.5. Factorial plan to evaluate the effect of different percentages of suitable emulsifier and EVOO on emulsion stability

A new factorial plan was elaborated to evaluate the effect of different percentages of the suitable emulsifier chosen and EVOO. The emulsions were prepared with five percentages of suitable emulsifier (12; 15; 18; 22 and 25%) and of EVOO (10; 20; 30; 40 and 50%). Coded and actual values of variables are shown in **Table D2** (**Appendix D**). The factorial design of two variables (percentages of emulsifier and EVOO) and five levels was also elaborated according to CCD (Box *et al.*, 1978). Eleven tests with different combinations of variable values were obtained (**Table 8**). All the emulsions were prepared as described in ***"section 1.4."***, with the amounts defined at the factorial plan (**Table 8**).

Table 8 - Experimental factorial plan elaborated to evaluate the effects of different percentages of emulsifier and EVOO on viscosity of obtained emulsions. Values with an * represent the central point.

Samples	Emulsifier in water phase (%)	EVOO (%)
1	15	20
2	15	40
3	22	20
4	22	40
5	13	30
6	23	30
7	18	16
8	18	44
9	18*	30*
10	18	30
11	18	30

1.6. Analyses

1.6.1. Chemical composition of EVOO

In a preliminary investigation, aiming to characterize the different oils used regarding their polyphenol content and their physical parameters and chemical composition, the samples were analysed using a NIR spectrometer model XDS Rapid Content^{TM} Analyser (FOSS, Hilleroed, Denmark). This instrument is equipped with a 400-2500 nm monochromator, temperature control of the analytical module (± 0.1 °C) and work on the sample as it is, without any preparatory phase. The specific glass cuvette was filled with 2 mL of each sample.

1.6.2. pH determination

Using a pH meter model HI 8424 (Hanna Instrument, Padova, Italy), the pH was measured on the water phase of the emulsion. The measurements were made at 25 °C by direct immersion of pH meter glass electrode on the samples.

1.6.3. Water activity determination

The a_w values of emulsions were measured with a dew point hygrometer at 25 °C (Aqualab CX-2, Decagon Devices Inc.TM, Washington, USA).

1.6.4. Electrical conductivity and solubility test

Electrical conductivity and solubility tests of the emulsions were performed in order to characterize the type of emulsion produced by ultrasound treatment. The electrical conductivity was measured using a conductivity meter COND 7 with microprocessor complete, cell 2301T, 2 electrodes and built-in temperature (Chemie, Valenzano, Italy), being the measurements made at 25 °C by direct immersion of glass electrode in samples.

The solubility tests were carried out by mixing 10 g of emulsion in 100 mL of different solvents, with different polarity: distilled water, ethanol, methanol and hexane for 60 minutes.

1.6.5. Density determination

Density was determined by weighing 10 grams of emulsions on a graduated cylinder. The density was calculated as ratio between mass and volume of the emulsion.

1.6.6. Accelerated emulsion stability by centrifugation

Accelerated stability of emulsions was determined according to the method of Huang *et al.* (2001), with some modifications. Freshly made emulsions were centrifuged using a centrifuge model PK120R (ALC, Cologno Monzese, Italy). Each emulsion (40 g) was placed into a 50 mL centrifugal plastic tube and centrifuged at 10,000 rpm for 10 min at 20 °C. Centrifugation produced three separate layers, an aqueous layer (bottom), emulsion layer (middle) and oil layer (top). Emulsions that yield the oil layer were considered unstable. The initial height of the emulsion (H_T) and the height of the emulsion layer (*He*) after centrifugation were measured.

Emulsion stability (*ES*) was calculated, as a percentage, with the following formula:

$$ES = \frac{H_e}{H_T} x100$$

1.6.7. Effect of centrifugal stress

Emulsions were centrifugated as reported in ***"section 1.6.6"*** and the creaming index of each emulsion was calculated using the following formula:

$$CI = 100 \times \frac{V_s}{V_t}$$

Where *CI* is creaming index, V_t is total volume of sample and V_s is volume of the lower phase layer.

1.6.8. Dynamic viscosity

The dynamic viscosity of emulsions was measured using a Zwick/Roell Z010 model BT1-FR010TH.A50 universal testing machine (ZwickRoell GmbH, Ulm, Germany) equipped with a back-extrusion chamber. During the measurements, a Plexiglass chamber with a height of 60 mm and an internal diameter of 85 mm, and a cylindrical piston having 75 mm diameter and 8 mm of height were used. The dynamic viscosity was determined by measuring five cycles (100, 200, 300, 400 and 800 $mm.min^{-1}$) in which the piston went through a 20 mm distance in each up-down cycle. The mean dynamic viscosity value was calculated based on the five cycles. The standard distance where was applied the load for each cycle was 20 mm while the standard travel where the load was removed was 0 mm. To perform the test, 40 g of each sample was used, and all tests were carried out at room temperature. The results obtained during the fivefold back extrusion tests were analysed using testXpert II v. 3.2. software especially developed for viscosity testing.

1.6.9. Microscopy analyses

Two different microscopes were used to observe emulsions' structure, an optical microscope and a scanning electron microscope (SEM).

The observations on the optical microscope were carried out by coating the glassy flat with each emulsion, without any previous treatment, and placing it on the stage of a light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The images of emulsions microstructure were obtained at a 40x magnification by a digital camera (Canon EOS 40BC, Pordenone, Italy) connected to the microscope.

The SEM (Hitachi M3030, Tokyo, Japan) was used to observe the surface morphology of emulsions. Emulsions were put on carbon adhesive tabs and then were covered by a thin layer of gold after sputtering under vacuum to prevent surface charging in the electron beam (Jadhav *et al.*, 2015). The different steps on the preparation of samples for SEM analysis are reported in **Figure 15**.

Figure 15 – Representation of the different steps for the preparation of samples for SEM analysis.

1.6.10. High pressure treatment of samples

In order to study the influence of HPP on emulsion's total phenolic content and microbiology, six and three grams of each sample, respectively, in triplicate, were aseptically individually packed, in UV-light sterilized, low permeability PA/PE bags (90 micron, Plásticos Macar – Indústria de Plástico Lda., Palmeira, Portugal), and manually heat-sealed, excluding as much air as possible.

HPP treatment was carried out according to the method described in ***"section 1.3 – Part I"*** for dairy cream, with some modifications. For this treatment, the emulsions were subjected to 500 MPa during 5 min.

After processing, all samples were stored at room temperature for 20 days to evaluate the growth of the studied microorganisms since day 0. Day 0 was set as first day immediately after high pressure treatment.

1.6.11. Total polyphenols content

Emulsions total phenolic content was determined before and after HPP treatment using the Folin-Ciocalteu spectrophotometric assay (Singleton and Rossi, 1965), by

mixing 5 g of each sample with 25 mL of a water:methanol (2:8) solution in a stomacher strainer bag, followed by homogenization for 3 min in a Stomacher 80 Biomaster, and then centrifugation (Heraeus Biofuge Stratos centrifuge, Thermo Electron corporation, Waltham, Massachusetts, United States) at 5 °C and 10,000 rpm for 10 min. The pellet was discarded, and the supernatant was retained and used as extract. Each extract (100 µL) was mixed with 1.58 mL water, 100 µL of Folin–Ciocalteau's reagent and 300 µL of sodium carbonate solution (200 g L^{-1}). After 2 h standing in the dark, the absorbance of the mixture was read at 725 nm using a UV-Vis spectrophotometer (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA). Seven standards of gallic acid were prepared with concentrations between 0 and 400 mg/L, being the total phenolic content expressed as mg of gallic acid equivalents per g of emulsion (mg gallic acid/g). All tests were performed in triplicate.

1.6.12. Microbiology

Emulsions made with the 3 EVOO cultivars (Arbequina, Coratina and Peranzana) were analysed for counts of total aerobic mesophiles (TAM) and yeasts and moulds (YM), which are naturally occurring microorganisms in olive oil (Palumbo and Harris, 2011), before, immediately after HPP treatment and after 20 days of storage at room temperature (15.83 ± 1.52 ºC). One gram of each sample, obtained aseptically, was homogenized with 9.0 mL of Ringer's solution for 240 seconds using a Stomacher at high-speed. Further, decimal dilutions were made with the same diluent and plated in triplicate on the appropriate media.

1.6.12.1. Total aerobic mesophiles counts

Total aerobic mesophiles counts were enumerated using 1.0 mL of diluted solution sample, on plate count agar (PCA, Merck), by incubation at 30 °C for 3 days (ISO 4833:2003) (International Organization for Standardization, 2003) under aerobic conditions.

1.6.12.2. Yeasts and moulds counts

Yeasts and moulds were enumerated using Rose Bengal chloramphenicol agar (RBCA; Merck) at 25 °C for 5 days (ISO 7954:1987) (International Organization for Standardization, 1987). These microorganisms were plated using the spread-plate method with 200 µL per sample, being counted pink colonies of yeasts and moulds.

1.6.13. Microbial counts

Petri dishes containing 15-300 colonies forming units (CFU) were selected for counting of TAM, and 15-150 colonies for YM counting. The obtained results were expressed as decimal logarithm of colony forming units per gram of emulsion (Log CFU/g). The maximum endogenous microbial load considered in this study was 6.00 log CFU/g (Institute of Medicine (US) and National Research Council (US) Committee, 2003), while the detection limit for this method was 1.00 log CFU/g. Microbial counts were calculated using the **Equation 1**, on the previous section.

1.6.14. Statistical analysis

Data were submitted to statistical analysis using Statsoft, version 5.1 (Statsoft, Tulsa, USA) software. The analysis was carried out in two steps. The first involved a stepwise regression analysis to identify the relevant variables, and the second used a multiple regression analysis (Standard Least Square Fitting) to fit a second order mathematical model, according to the following polynomial equation **(Equation 3)**:

$$\mathbf{y = B_0 + \Sigma B_{i\chi i} + \Sigma B_{ii\chi ii2} + \Sigma B_{ij\chi i\chi j}} \qquad \textbf{(Equation 3)}$$

where y is the dependent variable, B_0 is a constant value, χi and χj are the independent variables in coded values and B_i, B_{ii} and B_{ij} are the regression coefficients of the model. This model allowed the effects of the linear (χi), quadratic (χi^2) and combined ($\chi i \chi j$) terms of the independent variables to be assessed on the dependent variable.

Variables with a significance lower than 95% (p>0.05) were left out of the equation. Iso-response surface was developed in order to describe both individual and interactive effects of the independent variables on analytical indexes.

Analysis of variance (ANOVA) was performed using StatSoft software version 6.0 (Statsoft, Oklahoma, USA) to evaluate the effects of different types of oil on the analytical indexes used to characterize the emulsions. The mean values were compared by Fisher's test.

PART I

CARRIED OUT IN AVEIRO, PORTUGAL

CHAPTER III – RESULTS AND DISCUSSION

THIS SECTION REPORTS ALL THE OBTAINED RESULTS REGARDING THE EFFECT OF DIFFERENT PASTEURIZATION CONDITIONS ON CREAM'S MICROBIOLOGY AND GENERAL PROPERTIES AND COMPOSITION

1. Microbial analyses

In this study, the total aerobic psychrophiles, *Enterobacteriaceae*, lactic acid bacteria, *Escherichia coli* and *Listeria innocua* microbial counts were determined through all the storage conditions tested.

1.1. Total Aerobic Psychrophiles analysis

This group is considered the most relevant spoilage microorganisms in the dairy industry due to their capacity to grow and multiply during refrigerated storage (Decimo *et al.*, 2006). Unfortunately, cold and extended storage of raw milk is a common practice in the dairy sector, which favours the growth of these bacteria (de Oliveira *et al.*, 2015). In addition to the ability to grow at low temperatures, these microorganisms are also capable of producing heat-resistant extracellular enzymes (proteinases and lipases). Many of these enzymes retain their activity even after the conventional heat treatment of milk, being responsible for quality issues and sensory defects, which in turn results in a limited shelf-life of milk and dairy products (Decimo *et al.*, 2006; Samaržija *et al.*, 2012). *Pseudomonas* spp. and *Bacillus* spp., are among other psychrotrophic bacteria, associated with milk and dairy products, being the most common isolated organisms in raw or heat treated milk at the time of spoilage (Samaržija *et al.*, 2012).

Considering that refrigerated milk is almost exclusively used for the production of cream, psychrotrophic bacteria can be considered the main cause of spoilage and reduced shelf-life of this product. Since cream is a high fat content product, it is prone to lipolytic spoilage caused by the already mentioned thermostable enzymes of these bacteria (Samaržija *et al.*, 2012).

Regarding the first batch of cream, TAP was quantified before (initial) and right after thermal or HPP (day zero), and also at the 5th, 9th, 18th, 33rd and 51st days of refrigerated storage (4 °C) (**Figure 16**). The initial TAP load of cream was 4.36 ± 0.03 log CFU/mL, similar to Ribeiro Júnior *et al.* (2017) who reported a mean count of 4.04 log CFU/mL in raw milk.

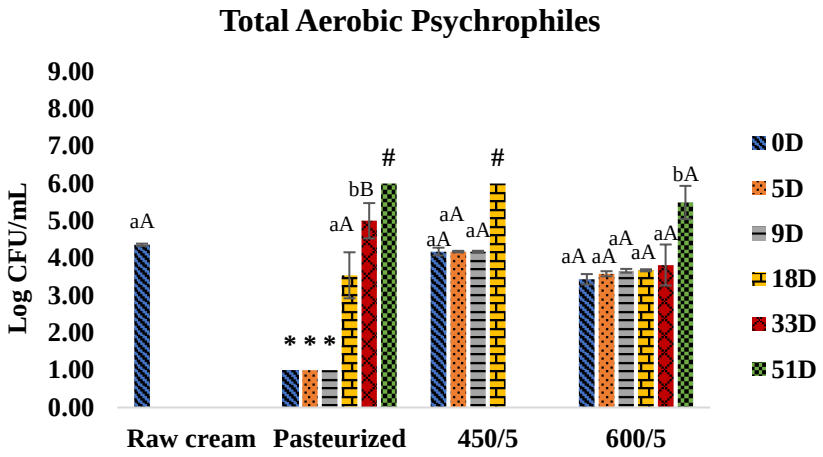


Figure 16 - Microbial growth of TAP on initial raw cream, after heat treatment and pressure treatment under 450 MPa and 600 MPa during 5 min. Analyses were made on initial cream and right after processing (0D) and on day 5 (5D), 9 (9D), 18 (18D), 33 (33D) and 51 (51D) of storage at 4 °C. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Different letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-b).

As represented in **Figure 16**, TAP counts were decreased to below the detection limit (≤ 1.00 log CFU/mL) right after thermal pasteurization (0D), maintaining these low counts until the 9th day of storage. From the 18th day onwards, TAP counts present on thermally pasteurized cream samples increased significantly (p<0.05), surpassing 6.00 log CFU/mL (maximum load considered) by the 51st day of refrigerated storage.

Samples processed at 450 and 600 MPa for 5 min presented a similar inactivation effect (**Figure 16**), with a reduction (p>0.05) on TAP counts of about 1.04 and 1.27-fold, respectively, comparatively to initial raw cream counts. Until the 9th day of storage, TAP counts for 450/5 cream samples remained practically unchanged at values around 4.18 log CFU/mL (p>0.05). However, from the 18th day onwards TAP counts were above the established limit of 6.00 log CFU/mL, thus being considered as spoiled cream. TAP recovery after the 600/5 treatment was slower in comparison to the 450/5 treatment, since its counts only increased significantly (p<0.05) after 51 days of storage, reaching counts of 5.49 ± 0.45 log CFU/mL. Furthermore, after 33 days of storage, TAP counts were significantly lower (p<0.05) on 600/5 samples when compared to the thermally pasteurized ones. Moreover, as aforementioned, by the 51st day of storage, TAP counts from thermally pasteurized samples had already surpassed 6.00 log CFU/mL, while those from 600/5 were still below this acceptable limit.

In order to further and deeply evaluate the influence of time on HPP effects, a second set of experiments was performed. For this second study, new fresh cream samples were

exposed to the same pressure level, 600 MPa, but for a longer time, 15 min (600/15), and the effect on creams' microbial load was also studied. Since the new raw cream samples were from a different batch, it was necessary to access the initial microbial load, being the value obtained of 4.67 ± 0.03 log CFU/mL for TAP counts, slightly higher (p<0.05) than the one previously obtained (**Figure 17**).

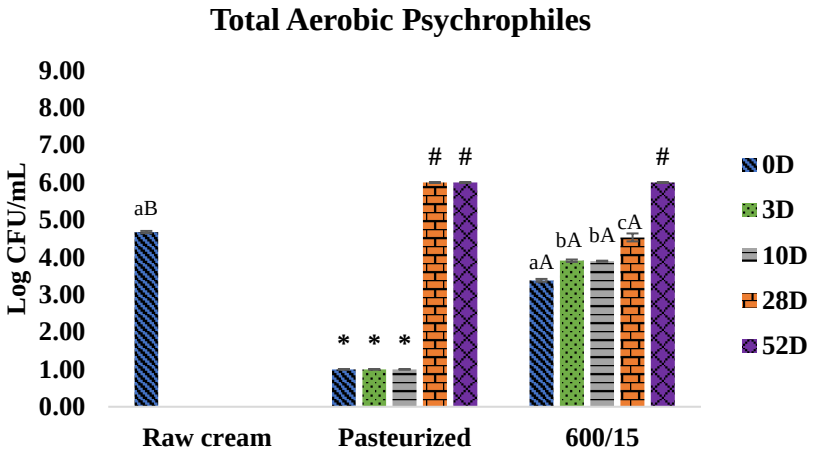


Figure 17 - Microbial growth of TAP on initial raw cream, after heat treatment and pressure treatment under 600 MPa for 15 min. Analyses were made on initial cream and right after processing (0D) and on day 3 (3D), 10 (10D), 28 (28D) and 52 (52D) of storage at 4 °C. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Different letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-c).

The 600/15 condition significantly reduced (p<0.05) TAP counts of about 1.38-fold, when compared to initial raw cream counts. By the 28th day of storage, TAP counts from thermally pasteurized samples increased to values above 6.00 log CFU/mL, being considered unacceptable for consumption, while those pasteurized by HPP (600/15) presented counts of 4.53 ± 0.11 log CFU/mL, only surpassing 6.00 log CFU/mL after 52 days of storage. Like 600/5, a longer exposure time to the same pressure value showed to be more effective at inhibiting long-term microbial development when compared to thermal pasteurization.

1.2. *Enterobacteriaceae* analysis

The *Enterobacteriaceae* (ENT) family is constituted by a large group of Gram-negative, non-spore-forming and facultative anaerobes. Its family includes members of the coliform group (including *E. coli*), comprising also a number of important foodborne pathogens such as *Salmonella*, *Shigella* and *Yersinia*, which are responsible for the spoilage of a variety of foods. Usually, *Enterobacteriaceae* are isolated from food as

evidence of poor hygiene or inadequate processing (especially heat-treatment), process failure and post-process contamination (Baylis *et al.*, 2011; Lehner and Stephan, 2016).

The initial ENT load in raw cream was 2.47 ± 0.05 log CFU/mL, as it can be seen in **Figure 18**.

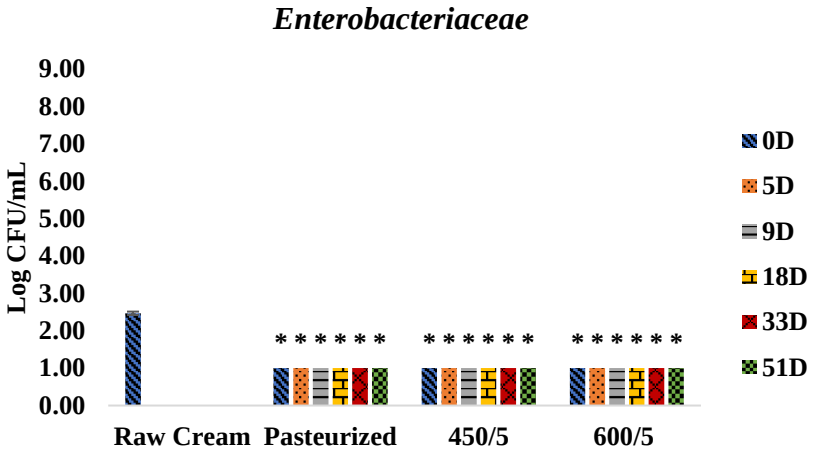


Figure 18 - Microbial growth of *Enterobacteriaceae* on initial raw cream, after heat and pressure treatment under 450 MPa and 600 MPa during 5 min. Analyses were made on initial cream and right after processing (0D) and on day 5 (5D), 9 (9D), 18 (18D), 33 (33D) and 51 (51D) of storage at 4 °C. Bars with * represent microbial loads below the detection limit (lower than 1.00 log CFU/mL).

Compared with the other microorganisms studied, ENT was the most affected by all conditions. As represented in **Figure 18**, ENT counts from thermally pasteurized and pressurized (450 and 600 MPa for 5 min) cream samples were reduced to values below the detection limit, maintaining these values throughout the entire storage period. These results are in agreement with Permanyer, Castellote, Audí, and Castell (2010), that reported a similar barosensivity of ENT when human milk was pressurized at 400, 500 and 600 MPa for 5 min at 12 °C.

Since on this first study ENT exhibited a high sensitivity to both high pressure and thermal pasteurization, the effect of the 600/15 condition was not evaluated on this microorganism.

1.3. Lactic Acid Bacteria analysis

Lactic acid bacteria (LAB) are a highly heterogeneous bacterial group, being acid-tolerant gram-positive bacteria, non-sporulating rods or cocci, able to produce lactic acid during homo or heterofermentative metabolism (Burgain *et al.*, 2014). They have been widely applied in food fermentation (dairy and non-dairy fermentations) worldwide due to their known status as generally recognized as safe (GRAS). Milk and fermentative milk products are favorable substrates for the growth of spoilage microorganisms. Thus, since

one of the most important properties of LAB is their ability to produce acid, they exhibit antimicrobial activity, protecting milk against spoilage microorganisms and proliferation of pathogens (Widyastuti *et al.*, 2014).

Regarding the first batch, the initial LAB counts of raw cream was 4.01 ± 0.05 log CFU/mL (**Figure 19**), similar to the maximum LAB count (4.20 log CFU/mL) obtained by Desmasures, Bazin and Guéguen (1997) for raw milk.

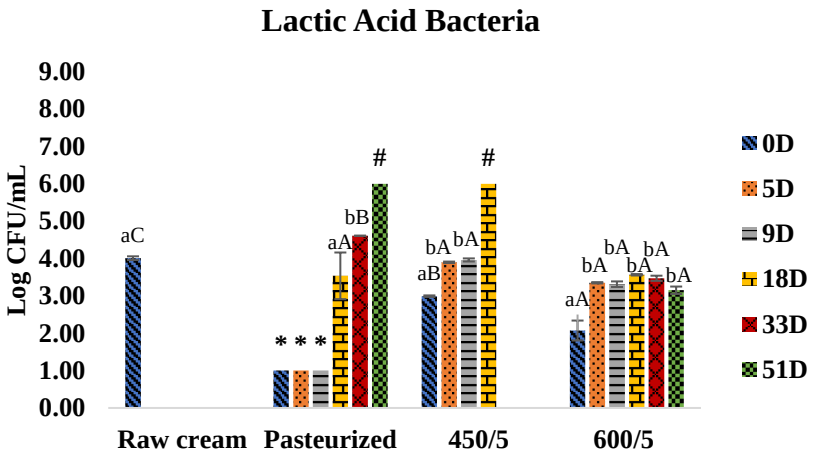


Figure 19 - Microbial growth of LAB on initial raw cream, after heat treatment and pressure treatment under 450 MPa and 600 MPa during 5 min. Analyses were made on initial cream and right after processing (0D) and on day 5 (5D), 9 (9D), 18 (18D), 33 (33D) and 51 (51D) of storage at 4 °C. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Different letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-b).

After both thermal and HPP, LAB and TAP counts presented a quite similar behaviour throughout the storage period. Likewise, LAB counts also decreased to below the detection limit after cream samples were thermally pasteurized, when compared to the initial raw cream load. Its counts remained undetectable until the 9th day of storage, and like TAP, it was only from the 18th day onwards that LAB counts experienced a significant increase (p<0.05), surpassing 6.00 log CFU/mL by the 51st day of refrigerated storage for the pasteurized cream samples. Since TAP and LAB counts surpassed the maximum load considered on the 51st day, a decision was made to do not proceed further with thermally pasteurized cream analysis.

Concerning both HPP treatments (450/5 and 600/5), they were able to significantly decrease LAB counts (p<0.05) of about 1.34 and 1.94-fold, comparatively to the initial raw cream counts. The 600/5 treatment had a more pronounced effect, meaning that, in this case, higher pressure resulted in a larger destruction of microorganisms, which was also observed by Pandey, Ramaswamy, and Idziak (2003).

While TAP counts of 450/5 cream remained practically unchanged (p>0.05) until the 9th day of storage, LAB counts increased significantly (p<0.05) after 5 days (**Figure 19**). From the 18th day onwards LAB counts, were above 6.00 log CFU/mL, thus being considered as spoiled cream. As expected, LAB recovery after 600/5 treatment was slower when compared to 450/5, remaining generally unchanged (p>0.05) from the 5th until the end of the storage period (**Figure 19**). Furthermore, after 33 days, similarly to what was observed for TAP counts, LAB counts (3.47 ± 0.07 log CFU/mL) were significantly lower (p<0.05) when compared to thermally pasteurized samples. Moreover, while LAB counts on thermally pasteurized samples had already surpassed 6.00 log CFU/mL by the 51st day, their counts on 600/5 were successfully lower, 3.15 ± 0.10 log CFU/mL.

Like for TAP, the initial value of LAB from the second batch was slightly higher, 4.40 ± 0.02 log CFU/mL (**Figure 20**). The 600/15 condition significantly reduced (p<0.05) LAB counts of about 1.77-fold, when compared to initial raw cream counts. Despite the fact that on the subsequent days of storage a significant increase (p<0.05) of LAB counts was observed, by the 52nd day LAB counts were still below 6.00 log CFU/mL, successfully exceeding literature's expected cream shelf-life of < 3 weeks.

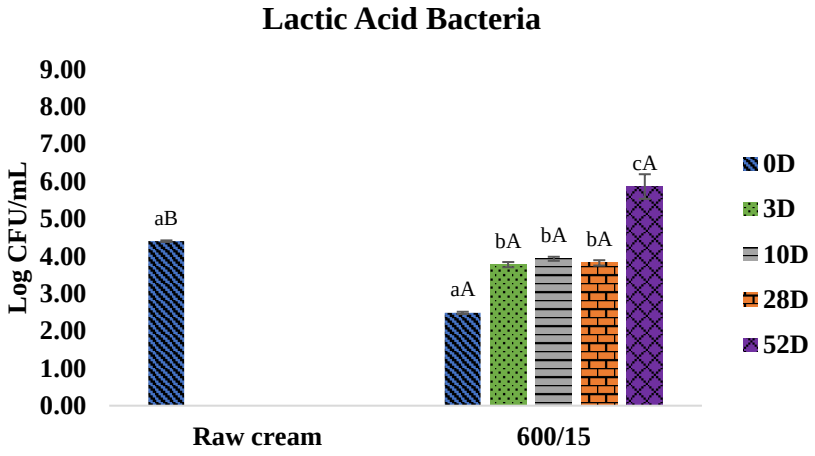


Figure 20 - Microbial growth of LAB on initial raw cream and after pressure treatment under 600 MPa for 15 min. Analyses were made on initial cream and right after processing (0D) and on day 3 (3D), 10 (10D), 28 (28D) and 52 (52D) of storage at 4 °C. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Different letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-c).

1.4. *Escherichia coli* analysis

Many microorganisms can contaminate milk and its products, among these are *Escherichia coli* (Bali *et al.*, 2013). *E. coli*, which belongs to the *Enterobacteriaceae*

family, is considered to be a good index of direct or indirect contamination of faecal origin (Pandey *et al.*, 2003). Being one of the main inhabitants of the intestinal tract of most mammalian species, most *E. coli* are harmless, however, some are known to be pathogenic, responsible for causing food-borne related illness through ingestion of contaminated foodstuffs. Its presence in milk products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard (Pandey *et al.*, 2003).

In this study, *E. coli* was inoculated at a final concentration of around 10^8 cells/mL, and then the effect of HPP (600/5 and 600/15) was evaluate on this microorganism.

Both 600/5 and 600/15 treatments were able to significantly reduce (p<0.05) *E. coli* counts of about 3.00 log CFU/mL and to below the detection limit, respectively, when compared to the initial inoculated load (7.98 ± 0.02 log CFU/mL) (**Figure 21**). By the 3rd day, *E. coli* counts from 600/5 samples were still around 5.00 log CFU/mL, however, those from 600/15 samples, went from below detection limit (day 0) to 2.33 ± 0.15 log CFU/mL.

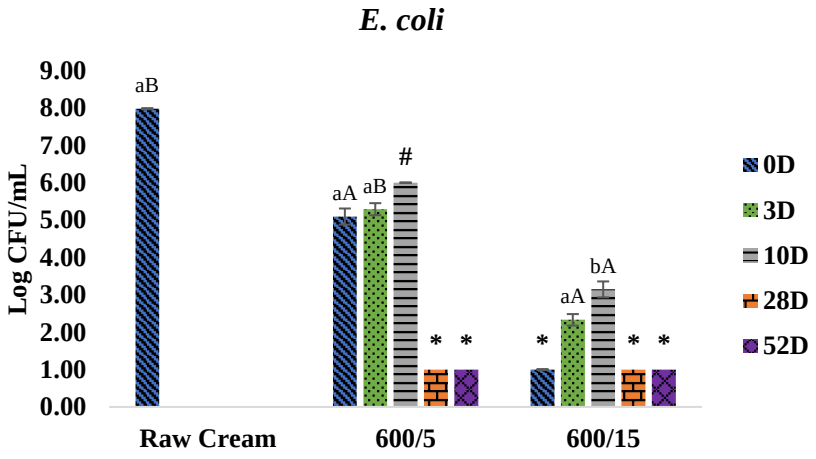


Figure 21 - Microbial growth of *E. coli* on initial raw cream and after pressure treatment under 600 MPa for 5 min and 600 MPa during 15 min. Analyses were made on initial cream and right after processing (0D) and on day 3 (3D), 10 (10D), 28 (28D) and 52 (52D) of storage at 4 °C. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Different letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-b).

E. coli counts from both 600/5 and 600/15 samples continued to increase until the 10th day, when it surpassed 6.00 log CFU/mL on 600/5 samples. However, on the following days, *E. coli* presented counts below the detection limit, remaining low until the end of the study. These results could probably be due to the fact that *E. coli* is not able to survive after long exposures to low temperatures. Arias, Monge-rojas, Chaves, and Antillón (2001) observed that *E. coli* O157:H7 populations decreased significantly (p<0.05) within

24 hours when milk that initially contained 10^8 CFU/mL was kept at 0 and 6 °C. However, after 48 h of storage at 6 and 12 °C, the authors detected an increase on *E. coli* counts. Furthermore, when stored at lower temperatures again, 0 and 6 °C, a significant ($p<0.05$) decrease was detected.

As expected, initially, a longer exposure to HPP was more effective on reducing *E. coli* counts to values below the detection limit, when comparing to the initially inoculated load, and on slowing its growth over time. However, in the end of the storage period, 600/5 and 600/15 treatments presented similar outcomes.

1.5. *Listeria innocua* analysis

Listeria monocytogenes, which has been described as the causative agent of foodborne illness associated with consumption of milk and other milk products, has the ability to grow under a wide variety of food-processing conditions. A nonhuman pathogen, *L. innocua* which is phylogenetic close to *L. monocytogenes*, was used as its surrogate (Gervilla *et al.*, 2016). And so, *L. innocua* was inoculated in raw cream, to see if HPP could significantly reduce *Listeria* counts in case cream was contaminated by *L. monocytogenes*.

L. innocua was inoculated in the second batch of cream samples at about 8.15 ± 0.02 log CFU/mL. Both 600/5 and 600/15 treatments allowed microbial inactivation to values below the detection limit until the 10^{th} day of storage (**Figure 22**). Similarly, Koseki, Mizuno and Yamamoto (2008) found out that *L. monocytogenes* cells inoculated in milk can be reduced to below the detection limit immediately after HPP above 550 MPa for 5 min.

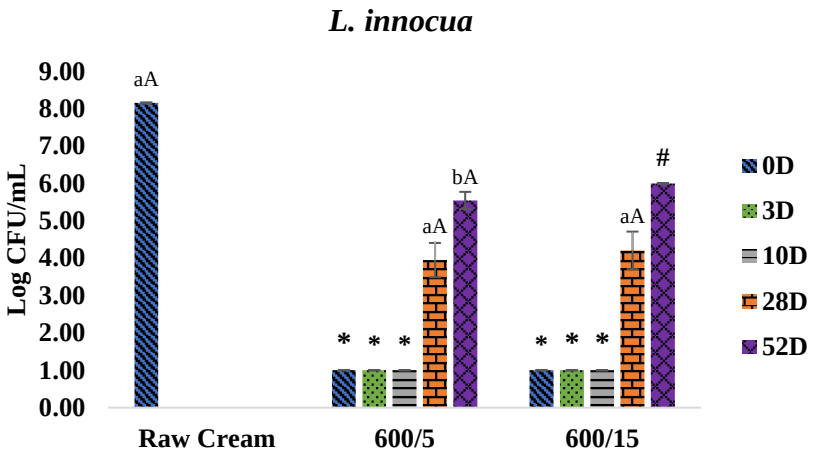


Figure 22 - Microbial growth of *Listeria innocua* on initial raw cream and after pressure treatment under 600 MPa for 5 min and 600 MPa during 15 min. Analyses were made on initial cream and right after processing (0D) and on day 3 (3D), 10 (10D), 28 (28D) and 52 (52D) of storage at 4 °C. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Upper/lower case letters denote significant differences (p<0.05) between storage days for each condition (A) and between treatment conditions for each storage day (a-b), respectively.

In general, previous works have revealed that gram-positive bacteria tend to be more resistant to HPP when compared to gram-negative bacteria (Trujillo, 2002; Viazis, Farkas, and Jaykus, 2008). In our study, by the 28th day of storage, *L. innocua* present in both 600/5 and 600/15 samples, grew and reached counts of 3.95 ± 0.46 log CFU/mL and 4.21 ± 0.50 log CFU/mL, respectively, continuing to increase (p<0.05) until the 52nd day. Koseki, Mizuno and Yamamoto (2008) also observed an increase in the number of *L. monocytogenes* cells in milk after 28 days of storage at 4 °C.

In the present study, possibly, HPP initially inactivated the majority of *L. innocua* load and also caused sublethal cellular damage, that over time could led to recovery and multiplication of this bacteria. The obtained results are in agreement with the conclusions of Bozoglu and Alpas (2004) that stated that damage caused by HPP may be repairable and that cells can potentially grow after repairing the site of injury during storage. The behaviour here observed for *L. innocua* was comparable to what Liepa *et al.* (2018) observed, indicating that the metabolic repair of gram-positive bacteria was more pressure resistant than that of gram-negative bacteria, in this case for *E. coli* ATCC 25922, since a large number of *L. monocytogenes* cells was observed after 10 days of milk's refrigerated storage.

1.6. General analysis

This is the first work that studied raw dairy creams behaviour when submitted to HPP and compared it with conventional thermal pasteurization, throughout a refrigerated storage period. Raffalli *et al.* (1994) studied the effect of HPP on *L. innocua* in liquid cream (35% fat), and demonstrated that it was possible to reduce considerably the microbial load of cream by HPP at 450 MPa and 25 °C for 10 to 30 min. In this study, the authors obtained a decimal reduction time of $D_{450\ MPa/25\ °C}$ = 7.4 min for *L. innocua*, while Gervilla, Ferragut, and Guamis (2000) obtained $D_{400\ MPa/25\ °C}$ = 4 min on ewe's milk (6% fat), showing the potential baroprotective effect of fat in microorganisms.

Overall, samples that underwent 600 MPa, gave better results than those processed at 450 MPa, both for 5 min, and especially than those that were thermally pasteurized, highlighting the efficiency of HPP at 600 MPa on microbial growth inhibition over time. In comparison, Stratakos *et al.* (2019) reported that HPP (600 MPa for 3 min) was able to significantly reduce total mesophilic aerobic bacteria, *Enterobacteriaceae*, LAB, and *Pseudomonas spp.*, prolonging milk microbial shelf-life by 7 days when compared to thermally pasteurized milk.

Taking into account the microbial stability observed after submitting samples to different pasteurization conditions, it was possible to conclude that even though thermal pasteurization was able to inhibit microbial growth on the first days of storage, when compared to HPP, the latter one managed to slow microbial growth over time, presenting less microbial counts on the last day of storage. According to Deosarkar *et al.* (2015), thermally pasteurized cream has a shelf-life of < 3 weeks at refrigeration temperature. Since microbiological results were equal to better than those obtained for thermally pasteurized samples, the present study suggests that HPP treatment of cream could be a promising alternative to thermal pasteurization, clearly pointing to a possible increase of cream's expectable shelf-life.

2. pH analysis

The initial pH-value of cream used in the first and second studies were similar to the ones reported in the literature, being 6.74 ± 0.05 and 6.91 ± 0.14, respectively (Dumay *et al.*, 1996; Gassi *et al.*, 2008). Regarding the first batch, cream samples presented small variations throughout refrigerated storage, for each condition (**Table 9**).

Table 9 - pH analysis of the different treatment condition (heat, 450/5 and 600/5) of the initial cream and right after processing (0D) and after 3 (3D), 10 (10D), 28 (28D) and 52 (52D) days of storage at 4 °C. Results are presented as mean ± standard deviation. Upper/lower case letters denote significant differences (p<0.05) between storage days for each condition (A) and between treatment conditions for each storage day (a-b), respectively.

Storage time (days)	Conditions	pH
0	Initial	6.74 ± 0.05^{aA}
	Heat-treated	6.93 ± 0.01^{aA}
	450 MPa/5 min	7.00 ± 0.02^{bA}
	600 MPa/5 min	6.86 ± 0.03^{abA}
5	Heat-treated	6.83 ± 0.02^{aA}
	450 MPa/5 min	6.95 ± 0.03^{bA}
	600 MPa/5 min	7.06 ± 0.12^{bA}
9	Heat-treated	6.89 ± 0.35^{aA}
	450 MPa/5 min	6.94 ± 0.20^{bA}
	600 MPa/5 min	6.86 ± 0.20^{abA}
18	Heat-treated	6.85 ± 0.18^{aA}
	450 MPa/5 min	6.66 ± 0.12^{aA}
	600 MPa/5 min	6.67 ± 0.05^{aA}
33	Heat-treated	6.64 ± 0.05^{aA}
	450 MPa/5 min	–
	600 MPa/5 min	6.66 ± 0.02^{aA}
51	Heat-treated	6.69 ± 0.22^{aA}
	450 MPa/5 min	–
	600 MPa/5 min	6.62 ± 0.05^{aA}

By looking at each condition individually, it was observed that the pH of thermally pasteurized samples remained stable throughout the storage period, being statistically similar (p>0.05) to the initial pH-value of raw cream. As for 450/5 samples, immediately after this treatment, a non-significant increase (p>0.05) of cream's pH was observed, when compared with raw cream. High pressure treatment can alter the distribution of minerals, due to casein micelle disaggregation. In Liepa, Zagorska, and Galoburda (2017)'s study, the increase in the concentration of phosphate in milk serum, caused an increase in processed milk's pH when compared to raw milk. 450/5 samples were able to maintain their pH-values between 6.94 ± 0.20 and 7.00 ± 0.02 (p>0.05) until the 18th day, when the pH decreased significantly (p<0.05), probably caused by the observed microbial growth, due to organic acids production resulted from their metabolic activity (Decimo *et al.*, 2006). After this day, when samples were considered microbiologically unacceptable, a decision was made not to proceed with its physicochemical analysis. Dumay *et al*. (1996) worked with pressurized (450 MPa for 15-30 min) pasteurized cream and reported that the pH of creams remained stable after HPP until 7 days of refrigerated

storage at 4 °C. On the contrary, in this same study, pasteurized cream underwent apparent fermentation.

Under 600/5, when comparing to the initial pH value of raw cream, no considerable changes were detected (p>0.05).

Like on the first batch, on the second batch no significant differences (p>0.05) between treatments at each storage day were detected, except on the 3rd day when HPP samples had a significant higher pH-value than thermally pasteurized samples (**Table 10**).

Table 10 - pH analysis of the different treatment condition (heat and 600/15) of the initial cream and right after processing (0D) and after 3 (3D), 10 (10D), 28 (28D) and 52 (52D) days of storage at 4 °C. Results are presented as mean ± standard deviation. Upper/lower case letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-b), respectively.

Storage time (days)	Conditions	pH
0	Initial	6.91 ± 0.14^{aA}
	Heat-treated	6.75 ± 0.01^{bA}
	600 MPa/15 min	6.88 ± 0.05^{bA}
3	Heat-treated	6.66 ± 0.07^{bA}
	600 MPa/15 min	6.92 ± 0.08^{bA}
10	Heat-treated	6.44 ± 0.01^{aA}
	600 MPa/15 min	6.60 ± 0.10^{abA}
28	Heat-treated	6.66 ± 0.16^{bA}
	600 MPa/15 min	6.54 ± 0.03^{aA}
52	Heat-treated	–
	600 MPa/15 min	6.62 ± 0.02^{abA}

The highest pH observed for 600/15 samples was on the 3rd day (6.92 ± 0.08), and from the 10th day onwards, a significant decrease (p<0.05) on pH-values was observed, achieving the lowest value on the 28th (6.54 ± 0.03). This decrease over time can be related to the high microbial growth (both on TAP and LAB counts) observed on the 28th day, as mentioned before. Regarding thermally pasteurized samples, no significant variations were observed throughout the storage period (p>0.05), except on the 10th day, when pH-value experienced a significant decrease (p<0.05).

3. Colour analysis

Colour is one of the most important attributes of food (Hogan and Kelly, 2005), which highly influences consumer's decision, thus its preservation during storage is of paramount importance.

Similarly to pH measurement, colour analysis was only performed on microbiologically acceptable samples.

The initial cream values of *L**, *a** and *b** observed on the first batch were 47.3 ± 0.32, -0.05 ± 0.11 and 4.80 ± 0.32, respectively (**Table 11**), as for samples used on the second batch, initial *L**, *a** and *b** values were 51.0 ± 0.27, 0.42 ± 0.05 and 6.16 ± 0.27, respectively (**Table 12**).

Table 11 - Colour analysis of the different treatment condition (heat, 450/5 and 600/5) of the initial cream and right after processing (0D) and after 5 (5D), 9 (9D), 18 (18D), 33 (33D) and 51 (51D) days of storage at 4 °C. Results are presented as mean ± standard deviation. Upper/lower case letters denote significant differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-b), respectively.

Storage time (days)	Conditions	*L**	*a**	*b**	*ΔE**
0	Initial	47.3 ± 0.32^{aA}	-0.05 ± 0.11^{aA}	4.80 ± 0.32^{aA}	
	Heat-treated	48.5 ± 0.98^{aA}	-0.09 ± 0.15^{aA}	4.97 ± 0.98^{aA}	1.48 ± 0.60^{aA}
	450 MPa/5 min	49.0 ± 0.16^{aA}	0.31 ± 0.04^{aB}	5.04 ± 0.16^{aA}	2.19 ± 2.23^{aA}
	600 MPa/5 min	48.6 ± 0.08^{aA}	0.19 ± 0.04^{aB}	5.19 ± 0.08^{aA}	1.43 ± 1.00^{aA}
5	Heat-treated	50.9 ± 2.20^{aA}	0.11 ± 0.08^{abA}	6.67 ± 0.40^{bA}	4.12 ± 0.87^{aA}
	450 MPa/5 min	49.5 ± 0.85^{aA}	0.51 ± 0.02^{abB}	6.11 ± 0.16^{abA}	2.87 ± 2.26^{aA}
	600 MPa/5 min	51.5 ± 0.81^{abA}	0.47 ± 0.01^{bB}	6.58 ± 0.13^{bA}	4.85 ± 2.68^{aA}
9	Heat-treated	52.1 ± 1.70^{aA}	0.15 ± 0.08^{bA}	6.83 ± 0.30^{bA}	5.29 ± 1.77^{aA}
	450 MPa/5 min	51.4 ± 0.33^{aA}	0.60 ± 0.02^{bB}	6.58 ± 0.08^{bA}	4.64 ± 1.98^{aA}
	600 MPa/5 min	53.5 ± 2.37^{abA}	0.53 ± 0.03^{bB}	6.91 ± 0.32^{bA}	6.66 ± 1.58^{aA}
18	Heat-treated	52.7 ± 0.96^{aA}	0.17 ± 0.04^{bA}	6.92 ± 0.20^{bA}	5.88 ± 2.86^{aA}
	450 MPa/5 min	52.0 ± 0.75^{aA}	0.56 ± 0.04^{bB}	6.64 ± 0.08^{bA}	5.20 ± 1.55^{aA}
	600 MPa/5 min	53.3 ± 2.15^{abA}	0.41 ± 0.03^{abB}	6.88 ± 0.24^{bA}	6.40 ± 1.01^{aA}
33	Heat-treated	51.4 ± 0.96^{aA}	0.04 ± 0.03^{abA}	6.70 ± 0.13^{bA}	4.86 ± 2.51^{aA}
	450 MPa/5 min	–	–	–	–
	600 MPa/5 min	53.4 ± 1.40^{abA}	0.35 ± 0.07^{abB}	7.07 ± 0.24^{bA}	6.62 ± 1.00^{aA}
51	Heat-treated	53.6 ± 2.00^{aA}	0.17 ± 0.09^{bA}	7.26 ± 0.36^{bA}	8.01 ± 3.71^{aA}
	450 MPa/5 min	–	–	–	–
	600 MPa/5 min	55.2 ± 0.39^{bA}	0.54 ± 0.01^{bB}	7.45 ± 0.04^{bA}	8.43 ± 2.60^{aA}

Table 12 - Colour analysis of the different treatment condition (heat, 600/5 and 600/15) of the initial cream and right after processing (0D) and after 3 (3D), 10 (10D), 28 (28D) and 52 (52D) days of storage at 4 °C. Results are presented as mean ± standard deviation. Upper/lower case letters denote significant differences (p<0.05) between storage days for each condition (A-C) and between treatment conditions for each storage day (a-b), respectively.

Storage time (days)	Conditions	*L**	*a**	*b**	*ΔE**
0	Initial	51.0 ± 0.27^{aA}	0.42 ± 0.05^{aB}	6.16 ± 0.27^{aA}	
	Heat-treated	50.2 ± 0.12^{aA}	-0.08 ± 0.00^{aA}	6.15 ± 0.12^{aA}	1.40 ± 1.08^{aA}
	600 MPa/5 min	51.1 ± 0.03^{aA}	0.38 ± 0.03^{aB}	6.66 ± 0.02^{aA}	2.12 ± 0.83^{aA}
	600 MPa/15 min	50.7 ± 0.16^{aA}	0.25 ± 0.02^{abB}	6.63 ± 0.16^{aA}	2.51 ± 1.49^{aA}
3	Heat-treated	54.1 ± 2.06^{aA}	0.12 ± 0.08^{bA}	7.08 ± 0.40^{bA}	4.72 ± 2.15^{aA}
	600 MPa/5 min	51.5 ± 0.73^{aA}	0.39 ± 0.07^{aB}	6.51 ± 0.14^{aA}	1.47 ± 0.22^{aA}
	600 MPa/15 min	52.0 ± 0.48^{abA}	0.33 ± 0.06^{bB}	6.65 ± 0.05^{aA}	1.72 ± 0.91^{aA}
10	Heat-treated	52.4 ± 0.97^{aA}	0.06 ± 0.03^{abA}	6.80 ± 0.24^{abA}	3.09 ± 1.31^{aA}
	600 MPa/5 min	52.1 ± 0.48^{aA}	0.41 ± 0.03^{aB}	6.69 ± 0.10^{aA}	2.41 ± 1.15^{aA}
	600 MPa/15 min	52.0 ± 1.03^{abA}	0.35 ± 0.03^{bB}	6.79 ± 0.19^{aA}	2.94 ± 1.32^{aA}
28	Heat-treated	53.8 ± 1.50^{aA}	0.16 ± 0.05^{bA}	7.09 ± 0.26^{bA}	4.15 ± 1.57^{aA}
	600 MPa/5 min	53.9 ± 0.29^{aA}	0.49 ± 0.02^{aB}	7.03 ± 0.025^{aA}	3.03 ± 1.66^{aA}
	600 MPa/15 min	56.5 ± 3.11^{bA}	0.38 ± 0.05^{bB}	7.45 ± 0.40^{aA}	5.79 ± 4.78^{aA}

	Heat-treated	–	–	–	–
52	600 MPa/5 min	55.1 ± 2.36^{aA}	0.31 ± 0.01^{aB}	7.24 ± 0.30^{aA}	4.35 ± 3.03^{aA}
	600 MPa/15 min	53.6 ± 0.54^{abA}	0.13 ± 0.03^{aAB}	7.14 ± 0.04^{aA}	3.01 ± 2.00^{aA}

No significant differences (p>0.05) were found between untreated cream and right after processing under the studied conditions (day 0), for L^* and b^*, on both batches. The L^* parameter on both studies remained stable at all different storage days and conditions, except on 600/5 samples where a significant increase (p<0.05) was observed when comparing the value obtained immediately after processing with that obtained on the 51st day of storage.

The a^* parameter suffered some variations concerning both batches. On the first batch, when comparing to initial raw cream, it was significantly higher (p<0.05) for both HPP samples, and similar (p>0.05) to the thermally pasteurized. On the contrary, on the second study, initial a^* values of all samples, treated and non-treated, were statistically different (p<0.05) from each other, being the order from the highest a^* value to the lowest, the following: Raw cream > 600/15 > thermally pasteurized. These variations between the first and second studies are probably due to differences between cream's batch.

By looking at every storage period, 450/5 and 600/5 samples remained statistically similar between each other (p>0.05), differing only from thermally treated samples (p<0.05). The same happened with 600/15 and thermally treated samples on the second batch (p<0.05), being the a^* parameter on HPP samples always higher than the thermally treated.

The total colour change (ΔE^*) parameter is used to indicate the magnitude of colour difference between processed and unprocessed samples or before and after storage (Barba *et al.*, 2012; Stratakos *et al.*, 2019). Despite the small variations obtained for L^*, a^* and b^* parameters, no statistical differences were observed for the total colour change (ΔE^*), for all treatment conditions at each day of storage on both studies. Anyway, according to the classification method for perceiving these colour differences (Cserhalmi *et al.*, 2006), at the first days, noticeable colour differences were observed between all treatments and raw cream, and with increased storage time, these colour differences started to become more visible and greater. Data in the literature suggests that the processing of dairy food by high pressure may promote colour changes, for instance Stratakos *et al*. (2019) studied the use of HPP, at different pressure levels (400-600 MPa) and processing times (1-5 min), in raw milk processing. Comparatively with initial raw milk, at the beginning of

milk's shelf-life, the observed values of ΔE^* for both HPP and thermally treated milk were 2.82 and 2.95, respectively, similarly to what was observed for raw cream. Unfortunately, information available about the effect of HPP on cream's colour is still very scarce.

4. Rheology analysis

Rheological properties of cream are of great interest since cream is the starting point for the manufacture of butter and other cream products, such as coffee cream or whipping cream, for example (Prentice, 1972).

Cream flow behaviour was studied only for raw, thermally pasteurized, 600/5 and 600/15 samples, from the second batch. These pressure conditions were chosen due to their higher efficiency on reducing microbial load, when compared to 450/5 treatment. Generally, the samples showed a qualitatively similar non-Newtonian flow behaviour with apparent viscosity decreasing with shear rate (shear thinning) (**Figure E1 – Appendix E**). The observed behaviour was expected for an emulsion and is in accordance to Taylor *et al*. (1997) who reported that at temperatures below 35 °C, cream's behaviour is non-Newtonian as consequence of the physical state of fatty acids, and also to Donsì, Ferrari, and Maresca (2011) who evaluated the rheological behaviour of milk cream under pressure (400-500 MPa for 5-10 min at 25 °C), and reported that HPP milk cream also behaved as a non-Newtonian pseudoplastic fluid.

The apparent viscosity of the studied samples was compared at a constant shear rate of 33 s^{-1} (**Table 13**). No major differences on initial viscosity values were detected between raw cream and all treated samples. Furthermore, throughout all storage period, viscosity values of each sample did not suffer significant changes. Dumay *et al*. (1996) reported that after HPP, pasteurized cream samples did not suffer considerable changes in its flow behaviour after 7 days of storage at 4 °C. By comparing HPP and thermal treated samples, at each storage day, it was observed that pressure-treated samples presented always a higher viscosity than heat treated samples (**Table 13**).

In general, it was possible to conclude that HPP indeed induced some changes on cream's original flow behaviour by increasing its viscosity (**Table 13**), when compared to thermal pasteurization, which demonstrates HPP's potential to be successfully applied on the production of innovative dairy products.

Table 13 - Apparent viscosity values determined at a particular shear rate (33 s^{-1}), for initial raw cream and cream at different treatment condition (thermal pasteurization, 600/5 and 600/15) right after processing (0D) and after 3 (3D), 10 (10D), 28 (28D) and 52 (52D) days of storage at 4 °C.

Storage time (days)	Conditions	Viscosity (Pa.s)
0	Initial	0.015
	Heat-treated	0.015
	600 MPa/5 min	0.015
	600 MPa/15 min	0.016
3	Heat-treated	0.018
	600 MPa/5 min	0.031
	600 MPa/15 min	0.026
10	Heat-treated	0.017
	600 MPa/5 min	0.027
	600 MPa/15 min	0.026
28	Heat-treated	0.016
	600 MPa/5 min	0.028
	600 MPa/15 min	0.030
52	Heat-treated	–
	600 MPa/5 min	0.030
	600 MPa/15 min	0.034

5. Fatty acid analysis

Like for rheology analysis, creams fatty acid profile was studied only for raw cream, thermally pasteurized, 600/5 and 600/15 samples. To our knowledge, this is the first study that reports the differences between fatty acids concentration of thermally pasteurized and pressure treated cream upon storage. The GC analysis revealed the presence of twenty-nine fatty acids (results expressed in % of total fatty acids), being cream essentially rich in saturated (64.60 ± 0.31%) followed by monounsaturated (25.05 ± 0.11%) and with a small percentage of polyunsaturated fatty acids (4.35 ± 0.13%) (**Table 14**). The dominating acids present on cream samples include palmitic (C16:0, 24.50 ± 0.12%), oleic (C18:1c, 20.03 ± 0.11%) and myristic (C14:0, 11.25 ± 0.07%) acid. These results are in accordance with those described by Decimo *et al*. (2006) and Rutkowska, Bialek, Adamska, and Zbikowska (2015), who observed that, in general, the dominating FAs on cream were those of long (C-16; C-18) and intermediate (C-12; C-14) carbon chain and unsaturated C-18:1 and C-18:2.

In general, different treatment conditions did not affect significantly (p>0.05) saturated and monounsaturated FAs. Polyunsaturated FAs decreased significantly (p<0.05) on the 52nd day of storage, when compared to previous storage periods, for all treatment conditions. Regarding the main fatty acids present on cream, only palmitic acid (C16:0) presented significant differences among treatments and initial raw cream, being

present in a higher amount on the latter. Moltó-puigmartí, Permanyer, Isabel, and López-sabater (2011) studied the effect of HPP on the FA composition of human milk and reported that HPP did not change significantly the fatty acid proportions when compared to untreated samples. Also, a study carried out by Gervilla, Ferragut, and Guamis (2001) on FFA content in ewe's milk have showed that HPP between 100-500 MPa at 4, 25 and 50 °C did not increase FFA content significantly, which might be due to total or partial inactivation of the native lipoprotein lipase (LPL) of milk by pressure, or compositional and structural changes of the milk fat globule membrane. This phenomenon is of great interest to avoid off flavours derived from lipolytic rancidity in milk.

Table 14 - Cream fatty acids composition (% of total fatty acids), at different treatment condition (heat, 600/5 and 600/15) of the initial cream and right after processing (0D) and after 3 (3D), 10 (10D), 28 (28D) and 52 (52D) days of storage at 4 °C. Results are presented as mean ± standard deviation. Different letters represent statistically differences (p<0.05) between storage days for each condition (A-B) and between treatment conditions for each storage day (a-b).

	Day 0				Day 3			Day 10			Day 28			Day 52		
Fatty acids	Initial	Heat-treated	600 MPa/5 min	600 MPa/15 min	Heat-treated	600 MPa/5 min	600 MPa/15 min	Heat-treated	600 MPa/5 min	600 MPa/15 min	Heat-treated	600 MPa/5 min	600 MPa/15 min	Heat-treated	600 MPa/5 min	600 MPa/15 min
C4:0	3.47 ± 0.05^{aA}	3.01 ± 0.42^{aA}	3.29 ± 0.23^{aA}	3.40 ± 0.03^{aA}	3.19 ± 0.09^{aA}	3.26 ± 0.17^{aA}	3.12 ± 0.13^{aA}	3.15 ± 0.26^{aA}	2.89 ±0.66^{aA}	2.75 ± 0.36^{aA}	2.55 ± 0.02^{aA}	3.13 ± 0.21^{aA}	3.40 ± 0.02^{aA}	3.25 ± 0.04^{aA}	3.10 ± 0.03^{aA}	2.97 ± 0.03^{aA}
C6:0	2.78 ± 0.01^{aA}	2.56 ± 0.10^{aA}	2.69 ± 0.03^{aA}	2.64 ± 0.02^{aA}	2.68 ± 0.02^{aA}	2.72 ± 0.05^{aA}	2.72 ± 0.05^{aA}	2.73 ± 0.06^{aA}	2.69 ± 0.20^{aA}	2.55 ± 0.24^{aA}	2.38 ± 0.17^{aA}	2.56 ± 0.10^{aA}	2.65 ± 0.01^{aA}	2.61 ± 0.01^{aA}	2.60 ± 0.02^{aA}	2.59 ± 0.02^{aA}
C8:0	1.55 ± 0.01^{aA}	1.46 ± 0.02^{abA}	1.50 ± 0.02^{aA}	1.46 ± 0.02^{aA}	1.50 ± 0.01^{abA}	1.52 ± 0.02^{aA}	1.55 ± 0.01^{aA}	1.54 ± 0.02^{bA}	1.55 ± 0.07^{aA}	1.47 ±0.10^{aA}	1.38 ± 0.10^{aA}	1.44 ± 0.04^{aA}	1.47 ± 0.01^{aA}	1.46 ± 0.00^{abA}	1.46 ± 0.01^{aA}	1.47 ± 0.01^{aA}
C10:0	3.35 ± 0.02^{aA}	3.16 ± 0.02^{aA}	3.19 ± 0.04^{aA}	3.12 ± 0.04^{aA}	3.23 ± 0.02^{aA}	3.26 ± 0.05^{aA}	3.31 ± 0.03^{aA}	3.29 ± 0.03^{aA}	3.32 ± 0.11^{aA}	3.23 ± 0.17^{aA}	3.05 ± 0.14^{aA}	3.15 ± 0.02^{aA}	3.15 ± 0.01^{aA}	3.15 ± 0.01^{aA}	3.14 ± 0.04^{aA}	3.16 ± 0.01^{aA}
C10:1	0.39 ± 0.00^{aA}	0.37 ± 0.00^{abA}	0.38 ± 0.01^{aA}	0.37 ± 0.00^{aA}	0.38 ±0.00^{abA}	0.39 ± 0.01^{aA}	0.40 ± 0.01^{aA}	0.39 ± 0.00^{bA}	0.40 ± 0.01^{aA}	0.39 ± 0.02^{aA}	0.36 ± 0.02^{aA}	0.38 ± 0.00^{aA}	0.38 ± 0.00^{aA}	0.37 ± 0.00^{abA}	0.37 ± 0.00^{aA}	0.37 ± 0.00^{aA}
C12:0	4.04 ± 0.03^{aB}	3.73 ± 0.01^{aA}	3.74 ± 0.04^{aA}	3.69 ± 0.03^{aA}	3.78 ± 0.03^{aA}	3.80 ± 0.05^{aA}	3.83 ± 0.02^{abA}	3.82 ± 0.04^{aA}	3.87 ± 0.07^{aA}	3.91 ± 0.16^{bA}	3.68 ± 0.07^{aA}	3.76 ± 0.05^{aA}	3.72 ± 0.00^{abA}	3.72 ± 0.00^{aA}	3.72 ± 0.04^{aA}	3.75 ± 0.02^{abA}
C14:0	11.25 ± 0.07^{aA}	11.16 ± 0.04^{aA}	11.13 ± 0.05^{aA}	11.14 ± 0.06^{aA}	11.21 ± 0.03^{aA}	11.25 ± 0.12^{aA}	11.32 ± 0.01^{aA}	11.29 ± 0.13^{aA}	11.42 ± 0.12^{aAB}	11.74 ± 0.31^{bB}	11.08 ± 0.05^{aA}	11.33 ± 0.20^{aA}	11.19 ± 0.00^{aA}	11.24 ± 0.02^{aA}	11.18 ± 0.08^{aA}	11.28 ± 0.04^{aA}
C14:1t	0.35 ± 0.01^{aA}	0.37 ± 0.03^{aA}	0.33 ± 0.07^{aA}	0.22 ± 0.12^{aA}	0.34 ± 0.03^{aA}	0.31 ± 0.07^{aA}	0.38 ± 0.02^{aA}	0.37 ± 0.01^{aA}	0.28 ± 0.08^{aA}	0.37 ± 0.01^{aA}	0.37 ± 0.02^{aA}	0.27 ± 0.14^{aA}	0.35 ± 0.01^{aA}	0.20 ± 0.12^{aA}	0.35 ± 0.01^{aA}	0.34 ± 0.01^{aA}
C14:1	1.23 ± 0.00^{aA}	1.25 ± 0.01^{aA}	1.25 ± 0.01^{aA}	1.24 ± 0.01^{aA}	1.28 ± 0.02^{aA}	1.25 ± 0.02^{aA}	1.27 ± 0.01^{abA}	1.26 ± 0.01^{aA}	1.26 ± 0.01^{aA}	1.31 ± 0.03^{bA}	1.24 ± 0.00^{aA}	1.25 ± 0.02^{aA}	1.24 ± 0.00^{aA}	1.24 ± 0.00^{aA}	1.23 ± 0.01^{aA}	1.23 ± 0.00^{aA}
ai-C15:0	0.66 ± 0.00^{aA}	0.73 ± 0.01^{abB}	0.73 ± 0.00^{aB}	0.72 ± 0.00^{aB}	0.76 ± 0.02^{bB}	0.72 ± 0.01^{aA}	0.73 ± 0.00^{abAB}	0.73 ± 0.01^{abA}	0.73 ± 0.00^{aA}	0.75 ± 0.01^{bA}	0.73 ± 0.00^{abA}	0.73 ± 0.01^{aA}	0.72 ± 0.00^{abA}	0.72 ± 0.00^{aA}	0.72 ± 0.00^{aA}	0.72 ± 0.00^{aA}
C15:0	1.07 ± 0.00^{aA}	1.10 ± 0.01^{aA}	1.09 ± 0.01^{aA}	1.09 ± 0.01^{aA}	1.10 ± 0.00^{aA}	1.10 ± 0.01^{aA}	1.10 ± 0.00^{aA}	1.10 ± 0.01^{aA}	1.11 ± 0.00^{aAB}	1.14 ± 0.02^{bB}	1.11 ± 0.01^{aA}	1.11 ± 0.01^{aA}	1.09 ± 0.00^{aA}	1.10 ± 0.01^{aA}	1.10 ± 0.01^{aA}	1.10 ± 0.00^{aA}
i-C16:0	0.33 ± 0.01^{aA}	0.36 ± 0.01^{aB}	0.36 ± 0.00^{aB}	0.35 ± 0.00^{aB}	0.37 ± 0.01^{aB}	0.36 ± 0.01^{aAB}	0.35 ± 0.00^{aA}	0.36 ± 0.00^{aA}	0.36 ± 0.00^{aA}	0.37 ± 0.01aA	0.36 ± 0.00^{aA}	0.36 ± 0.00^{aA}	0.35 ± 0.00^{aA}	0.35 ± 0.00^{aA}	0.36 ± 0.00^{aA}	0.36 ± 0.00^{aA}
C16:0	24.50 ± 0.12^{aB}	23.74 ± 0.13^{aA}	23.66 ± 0.06^{aA}	23.73 ± 0.10^{abA}	23.63 ± 0.08^{aA}	23.77 ± 0.14^{aA}	23.70 ± 0.05^{aA}	23.75 ± 0.23^{aA}	24.00 ± 0.13^{aA}	24.20 ± 0.25^{bA}	24.06 ± 0.15^{aA}	23.92 ± 0.16^{aA}	23.76 ± 0.01^{abA}	23.84 ± 0.08^{aA}	23.82 ± 0.07^{aA}	23.92 ± 0.05^{abA}
C16:1T	0.52 ± 0.01^{aA}	0.55 ± 0.03^{aA}	0.54 ± 0.00^{aA}	0.54 ± 0.02^{aA}	0.54 ± 0.02^{aA}	0.53 ± 0.04^{aA}	0.56 ± 0.03^{aA}	0.55 ± 0.02^{aA}	0.55 ± 0.02^{aA}	0.54 ± 0.02^{aA}	0.56 ± 0.02^{aA}	0.54 ± 0.02^{aA}	0.55 ± 0.02^{aA}	0.52 ± 0.03^{aA}	0.54 ± 0.04^{aA}	0.52 ± 0.01^{aA}
C16:1C	2.75 ± 0.02^{aB}	2.63 ± 0.03^{aA}	2.62 ± 0.00^{aA}	2.63 ± 0.01^{aA}	2.70 ± 0.06^{aA}	2.61 ± 0.02^{aA}	2.63 ± 0.01^{aA}	2.63 ± 0.03^{aA}	2.63 ± 0.02^{aA}	2.67 ± 0.01^{aA}	2.65 ± 0.03^{aA}	2.63 ± 0.02^{aA}	2.61 ± 0.00^{aA}	2.66 ± 0.07^{aA}	2.62 ± 0.01^{aA}	2.61 ± 0.01^{aA}
ai-C17:0	0.83 ± 0.01^{aA}	0.87 ± 0.01^{aB}	0.87 ± 0.01^{aB}	0.88 ± 0.01^{aB}	0.87 ± 0.01^{aA}	0.87 ± 0.01^{aA}	0.87 ± 0.01^{aA}	0.88 ± 0.01^{aA}	0.87 ± 0.01^{aA}	0.88 ± 0.01^{aA}	0.88 ± 0.01^{aA}	0.87 ± 0.01^{aA}	0.87 ± 0.00^{aA}	0.87 ± 0.00^{aA}	0.87 ± 0.00^{aA}	0.87 ± 0.00^{aA}
C17:0	0.63 ± 0.00^{aA}	0.64 ± 0.00^{aA}	0.63 ± 0.00^{aA}	0.65 ± 0.01^{aA}	0.66 ± 0.01^{aA}	0.64 ± 0.00^{aA}	0.64 ± 0.01^{aA}	0.64 ± 0.01^{aA}	0.64 ± 0.01^{aA}	0.65 ± 0.03^{aA}	0.66 ± 0.02^{aA}	0.65 ± 0.01^{aA}	0.64 ± 0.00^{aA}	0.65 ± 0.01^{aA}	0.64 ± 0.01^{aA}	0.65 ± 0.00^{aA}
i-C18:0	0.32 ± 0.02^{aA}	0.31 ± 0.00^{aA}	0.31 ± 0.01^{aA}	0.31 ± 0.00^{aA}	0.31 ± 0.01^{aA}	0.31 ± 0.01^{aA}	0.32 ± 0.01^{aA}	0.33 ± 0.02^{aA}	0.31 ± 0.00^{aA}	0.31 ± 0.01^{aA}	0.32 ± 0.00^{aA}	0.32 ± 0.01^{aA}	0.31 ± 0.00^{aA}	0.31 ± 0.00^{aA}	0.31 ± 0.00^{aA}	0.31 ± 0.00^{aA}
C18:0	9.26 ± 0.05^{aA}	9.91 ± 0.06^{abB}	9.79 ± 0.06^{aB}	9.91 ± 0.08^{aB}	9.79 ± 0.04^{abA}	9.76 ± 0.11^{aA}	9.68 ± 0.06^{aA}	9.64 ± 0.09^{aA}	9.80 ± 0.22^{aA}	9.61 ± 0.33^{aA}	10.14 ± 0.12^{bA}	9.88 ± 0.09^{aA}	9.89 ± 0.02^{aA}	9.95 ± 0.04^{abA}	9.99 ± 0.01^{aA}	10.04 ± 0.03^{aA}
C18:1t	4.05 ± 0.02^{aA}	4.65 ± 0.06^{aB}	4.62 ± 0.03^{aB}	4.64 ± 0.04^{aB}	4.64 ± 0.02^{aA}	4.53 ± 0.03^{aA}	4.59 ± 0.01^{aA}	4.56 ± 0.05^{aA}	4.54 ± 0.09^{aA}	4.55 ± 0.13^{aA}	4.75 ± 0.06^{aA}	4.63 ± 0.05^{aA}	4.59 ± 0.01^{aA}	4.65 ± 0.03^{aA}	4.65 ± 0.05^{aA}	4.66 ± 0.02^{aA}
C18:1c	20.03 ± 0.11^{aA}	20.82 ± 0.15^{aA}	20.70 ± 0.12^{aA}	20.81 ± 0.05^{aA}	20.45 ± 0.09^{aA}	20.32 ± 0.17^{aA}	20.39 ± 0.10^{aA}	20.38 ± 0.21^{aA}	20.36 ± 0.43^{aA}	20.24 ± 0.58^{aA}	20.96 ± 0.23^{aA}	20.56 ± 0.19^{aA}	20.56 ± 0.03^{aA}	20.75 ± 0.04^{aA}	20.76 ± 0.08^{aA}	20.63 ± 0.09^{aA}
C18:2t	0.83 ± 0.07^{aA}	0.86 ± 0.04 ^{aA}	0.85 ± 0.03 ^{aA}	0.76 ± 0.14^{aA}	0.85 ± 0.08^{aA}	0.84 ± 0.06^{aA}	0.81 ± 0.04^{aA}	0.89 ± 0.08^{aA}	0.79 ± 0.04^{aA}	0.84 ± 0.02^{aA}	0.84 ± 0.09^{aA}	0.86 ± 0.02^{aA}	0.84 ± 0.02^{aA}	0.84 ± 0.03^{aA}	0.86 ± 0.02^{aA}	0.89 ± 0.05^{aA}
C18:2c	2.99 ± 0.05^{aA}	2.95 ± 0.01^{aA}	2.96 ± 0.01^{aA}	2.96 ± 0.01^{aA}	2.91 ± 0.01^{aA}	2.91 ± 0.03^{aA}	2.90 ± 0.01^{aA}	2.90 ± 0.02^{aA}	2.90 ± 0.06^{aA}	2.93 ± 0.10^{aA}	3.00 ± 0.03^{aA}	2.93 ± 0.02^{aA}	2.93 ± 0.01^{aA}	2.93 ± 0.03^{aA}	2.97 ± 0.04^{aA}	2.93 ± 0.01^{aA}
C18:3n-6	0.06 ± 0.01^{aA}	0.07 ± 0.01^{aA}	0.07 ± 0.01^{aA}	0.07 ± 0.01^{aA}	0.06 ± 0.02^{aA}	0.07 ± 0.00^{aA}	0.08 ± 0.01^{aA}	0.08 ± 0.01^{aA}	0.07 ± 0.00^{aA}	0.08 ± 0.01^{aA}	0.07 ± 0.01^{aA}	0.07 ± 0.00^{aA}	0.07 ± 0.00^{aA}	0.08 ± 0.00^{aA}	0.08 ± 0.01^{aA}	0.07 ± 0.01^{aA}
C18:3n-3	0.33 ± 0.00^{aA}	0.28 ± 0.00^{aA}	0.28 ± 0.01^{aA}	0.24 ± 0.07^{aA}	0.29 ± 0.00^{aA}	0.28 ± 0.00^{aA}	0.28 ± 0.00^{aA}	0.27 ± 0.00^{aA}	0.28 ± 0.01^{aA}	0.27 ± 0.01^{aA}	0.29 ± 0.00^{aA}	0.28 ± 0.01^{aA}	0.28 ± 0.00^{aA}	0.29 ± 0.01^{aA}	0.28 ± 0.00^{aA}	0.28 ± 0.00^{aA}
C20:0	0.19 ± 0.01^{aA}	0.21 ± 0.00^{aA}	0.21 ± 0.01^{aA}	0.22 ± 0.02^{aA}	0.22 ± 0.01^{aA}	0.20 ±0.01^{aA}	0.21 ± 0.01^{aA}	0.20 ± 0.00^{aA}	0.20 ± 0.01^{aA}	0.20 ± 0.01^{aA}	0.21 ± 0.00^{aA}	0.21 ± 0.00^{aA}	0.21 ± 0.00^{aA}	0.23 ± 0.02^{aA}	0.21 ± 0.00^{aA}	0.22 ± 0.01^{aA}
C20:1	0.22 ± 0.00^{aA}	0.24 ± 0.01^{bAB}	0.24 ± 0.00^{bAB}	0.25 ± 0.02^{bB}	0.25 ± 0.01^{bA}	0.24 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.23 ± 0.01^{bA}	0.25 ± 0.01^{bA}	0.24 ± 0.01^{bA}	0.23 ± 0.00^{bA}	0.16 ± 0.00^{aA}	0.17 ± 0.00^{aA}	0.17 ± 0.01^{aA}
C22:0	0.06 ± 0.00^{aA}	0.07 ± 0.00^{aA}	0.07 ± 0.00 ^{aA}	0.07 ± 0.00^{aA}	0.06 ± 0.02aA	0.07 ± 0.00aA	0.06 ± 0.00^{aA}	0.06 ± 0.00^{aA}	0.06 ± 0.00^{aA}	0.07 ± 0.00^{aA}	0.07 ± 0.00^{aA}	0.07 ± 0.00aA	0.07 ± 0.00aA	0.29 ± 0.00^{bA}	0.30 ± 0.00^{bA}	0.30 ± 0.00^{bA}
C24:0	0.15 ± 0.02^{aA}	0.10 ± 0.00^{aA}	0.10 ± 0.01^{aA}	0.11 ± 0.01^{aA}	0.16 ± 0.03^{bA}	0.14 ± 0.03^{aA}	0.12 ± 0.01^{aA}	0.11 ± 0.01^{abA}	0.08 ± 0.00^{aA}	0.09 ± 0.01^{aA}	0.10 ± 0.00^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.10 ± 0.01^{aA}	0.11 ± 0.01^{aA}	0.11 ± 0.02^{aA}
Saturated	64.60 ± 0.31^{aB}	63.26 ± 0.37^{aAB}	63.50 ± 0.19^{aAB}	63.65 ± 0.22^{aA}	63.61 ± 0.16^{aA}	63.96 ± 0.43^{aA}	63.78 ± 0.12^{aA}	63.76 ± 0.07^{aA}	64.04 ± 0.81^{aA}	64.06 ± 0.78^{aA}	62.91 ± 0.22^{aA}	63.75 ± 0.14^{aA}	63.75 ± 0.02^{aA}	64.12 ± 0.16^{aA}	63.88 ± 0.27^{aA}	64.07 ± 0.08^{aA}
Monounsaturated	25.05 ± 0.11^{aA}	25.79 ± 0.21^{aA}	25.64 ± 0.12^{aA}	25.74 ± 0.06^{aA}	25.37 ± 0.10^{aA}	25.32 ± 0.18^{aA}	25.44 ± 0.04^{aA}	25.33 ± 0.22^{aA}	25.32 ± 0.45^{aA}	25.26 ± 0.54^{aA}	25.91 ± 0.25^{aA}	25.52 ± 0.18^{aA}	25.46 ± 0.02^{aA}	25.63 ± 0.10^{aA}	25.61 ± 0.09^{aA}	25.47 ± 0.09^{aA}
Polyunsaturated	4.35 ± 0.13^{aA}	4.23 ± 0.06^{bA}	4.23 ± 0.07^{bA}	4.18 ± 0.05^{bA}	4.36 ± 0.18^{bA}	4.24 ± 0.17^{bA}	4.12 ± 0.03^{bA}	4.14 ± 0.05^{bA}	4.20 ± 0.19^{bA}	4.10 ± 0.14^{bA}	4.37 ± 0.03^{bA}	4.17 ± 0.06^{bA}	4.16 ± 0.02^{bA}	3.73 ± 0.03^{aA}	3.77 ± 0.06^{aA}	3.72 ± 0.01^{aA}

6. Volatiles analysis

Cream volatile profile was studied only for raw, thermally pasteurized, 600/5 and 600/15 samples, from the second batch.

A total of 43 different VOCs were identified in cream samples (**Table 15**).

Table 15 - Volatile compounds identified in cream according to chemical classes.

Alcohols	Acids	Ketones/Aldehydes	Alkanes/Alkenes		Lactones
Ethanol	Acetic acid	2-propanone	Hexane	Undecane	Butyrolactone
Butanol	Butanoic acid	3-metil-2-butanone	Heptane	Dodecane	δ decalactone
	Hexanoic acid	2-pentanone	Decane	4-ethyloctane	δ dodecalactone
	Octanoic acid	3-hydroxy-2-butanone	4-ethyloctane	2,6-dimethyl undecane	δ laurolactone
	Decanoic acid	2-nonanone	5-methylnonane	Tridecane	
	Nonanoic acid	2-undecanone	3-methylnonane	Octene	
		Undecanone	2,2,4,6,6-pentamethyl heptane	Tolueno	
		Butanal	3-metilheptane		
		Hexanal	Heptane, 4,4-dimethyl		
		Heptanal	2,2,4,4-tetramethyloctane		
		Nonanal	2,6-dimethyl undecane		
		Decanal	4-methyl decane		

Table 16 shows the chemical families of the VOCs, and the total volatile amount (identified and non-identified) detected. In general, there was a tendency of total volatiles to increase throughout storage. In raw cream, the most abundant were aliphatic hydrocarbons, with 51.6 ± 1.9 mg/100 mL, followed by aldehydes/ketones (19.5 ± 1.3 mg/100 mL), acids (16.4 ± 2.5 mg/100 mL) and lactones (0.2 ± 0.1 mg/100 mL), in smaller amounts.

Table 16 - Cream volatile profile (mg/100g equivalents of cyclohexanone), according to chemical classes, at different treatment condition (heat, 600/5 and 600/15) of the initial cream and right after processing (0D) and after 3 (3D), 10 (10D), 28 (28D) and 52 (52D) days of storage at 4 °C. Results are presented as mean ± standard deviation. Different letters represent statistical differences (p<0.05) between storage days for each condition (A-C) and between treatment conditions for each storage day (a-d).

Storage time (days)	Conditions	Alcohols	Acids	Aldehydes/Ketones	Aliphatic hydrocarbons	Lactones	Total volatiles
0	Initial	nd	16.4 ± 2.5^{aA}	19.5 ± 1.3^{aA}	51.6 ± 1.9^{aAB}	0.2 ± 0.1^{aA}	315.1 ± 26.9^{aAB}
	Heat-treated	3.2 ± 0.4^{aAB}	33.5 ± 4.7^{aAB}	36.9 ± 1.0^{aAB}	80.5 ± 0.8^{aB}	1.1 ± 0.1^{aB}	492.8 ± 36.7^{aB}
	600 MPa/5 min	4.1 ± 0.6^{aAB}	54.8 ± 3.3^{aB}	26.2 ± 4.6^{aAB}	41.6 ± 0.4^{aA}	0.4 ± 0.1^{aAB}	291.1 ± 11.4^{aA}
	600 MPa/15 min	6.6 ± 0.6^{aB}	52.6 ± 6.4^{aB}	47.5 ± 1.0^{bB}	6.9 ± 0.2^{aA}	0.4 ± 0.1^{aAB}	314.1 ± 10.7^{aAB}
3	Heat-treated	3.6 ± 0.3^{aA}	34.3 ± 2.8^{aA}	49.6 ± 10.2^{abB}	94.4 ± 2.8^{abA}	0.7 ± 0.1^{aA}	427.2 ± 64.5^{aA}
	600 MPa/5 min	5.2 ± 0.3^{aA}	114.2 ± 13.7^{cB}	42.0 ± 0.7^{abAB}	152.5 ± 3.8^{bB}	0.9 ± 0.1^{aAB}	493.9 ± 21.7^{bA}
	600 MPa/15 min	4.4 ± 0.7^{aA}	53.1 ± 0.7^{aA}	23.0 ± 0.8^{aA}	79.4 ± 2.9^{bA}	1.6 ± 0.1^{bB}	479.9 ± 58.2^{aA}
10	Heat-treated	4.1 ± 0.2^{aA}	26.1 ± 3.7^{aA}	60.5 ± 1.7^{bAB}	121.6 ± 3.2^{bA}	1.5 ± 0.3^{aA}	373.5 ± 35.8^{aA}
	600 MPa/5 min	6.6 ± 0.3^{aA}	84.4 ± 4.8^{bB}	70.8 ± 6.4^{bB}	220.2 ± 13.4^{cB}	2.4 ± 0.3^{bB}	703.2 ± 66.1^{cB}
	600 MPa/15 min	8.3 ± 0.2^{aA}	78.9 ± 4.12^{bB}	43.4 ± 8.0^{abA}	310.2 ± 6.8^{dC}	2.3 ± 0.2^{bAB}	782.4 ± 32.4^{bB}
28	Heat-treated	20.3 ± 5.0^{bB}	62.8 ± 9.8^{bA}	74.5 ± 11.8^{bA}	69.2 ± 5.7^{aA}	2.6 ± 0.2^{bA}	687.9 ± 37.8^{bA}
	600 MPa/5 min	5.9 ± 1.2^{aA}	152.5 ± 3.8^{dB}	73.9 ± 2.6^{bA}	346.5 ± 36.4^{dB}	4.9 ± 0.3^{cB}	1007.3 ± 49.9^{dC}
	600 MPa/15 min	4.6 ± 0.4^{aA}	220.7 ± 6.7^{cC}	71.4 ± 6.4^{cA}	83.3 ± 2.2^{bA}	5.6 ± 0.1^{cB}	797.6 ± 28.6^{bB}
52	Heat-treated	50.5 ± 3.7^{cB}	126.1 ± 8.9^{cA}	61.9 ± 12.7^{bB}	64.2 ± 4.2^{aA}	2.6 ± 0.2^{bA}	751.2 ± 56.4^{bA}
	600 MPa/5 min	4.1 ± 0.3^{aA}	167.1 ± 6.4^{dB}	52.2 ± 2.2^{bAB}	119.9 ± 8.1^{bB}	5.3 ± 0.5^{cB}	783.3 ± 30.2^{cA}
	600 MPa/15 min	6.1 ± 0.3^{aA}	307.0 ± 3.3^{dC}	36.2 ± 3.4^{abA}	224.2 ± 1.2^{cC}	5.7 ± 0.2^{cB}	881.1 ± 112.7^{bA}

Immediately after thermal and HPP treatments, a new class of compounds, alcohols, was detected on cream samples, presenting higher amounts on 600/15 samples, followed by 600/5 and, with smaller amounts, thermal treated samples (p>0.05). Alcohols can be produced by the reduction of their corresponding aldehydes and methyl ketones, through the activity of LAB dehydrogenases or by sugar fermentation (Juan *et al.*, 2010). Thus, since aldehyde composition of all treated samples was statistically higher (p<0.05) than that of raw cream, they presented alcohol compounds.

Until the 10th day of storage, alcohol compounds of HPP and thermally treated samples remained statistically similar (p>0.05). From the 28th until the last day of storage, thermally treated samples started to present significant higher (p<0.05) amounts of alcohols, when compared to HPP samples. Similarly, Chugh *et al.* (2014) studied the effect of thermal pasteurization on skim milk volatile composition, and observed that during refrigerated storage, alcohol concentration increased, as a result of the reduction of the corresponding carbonyl compounds.

Regarding acid compounds, their content on raw cream statistically increased (p<0.05) about 2.1-fold immediately after thermal treatment. Similarly Chugh *et al.* (2014) observed an increase on acids concentration (p<0.05) in raw milk as the intensity of thermal treatment increase. As for HPP an increase in approximately 3.3 times was observed after 600/5 treatment and about 3.2-fold after 600/15 (**Table 16**). Throughout storage, HPP samples acid content remained statistically higher (p<0.05) than thermal treated samples. Furthermore, Garrido *et al.* (2015) observed a relevant increase on carboxylic acid content of human milk, after processing at 400 MPa or 600 MPa for 6 min, concluding that this increase could be associated with the release of short-chain FA, resulting from the degradation of triglycerides (lipolysis). Like alcohols, acids may also be produced by fermentation of lactose. Furthermore, acids can also act as precursor molecules for a series of catabolic reactions, which can lead to the production of other flavour compounds such as alcohols, lactones, and methyl ketones (Juan *et al.*, 2010).

Concerning cream aldehyde/ketones content, initially, all treated samples were statistically similar to raw cream (p>0.05), except for 600/15 samples, who presented significantly higher levels (p<0.05) (**Table 16**). Vazquez-Landaverde *et al.* (2006) observed that at 25 °C, ketone concentration in milk processed under 620 MPa at 1, 3 or 5 min, was similar to that of raw milk. Despite the fact that ketones are naturally present in raw milk, most of them can be formed during heat treatment by the β-oxidation of

saturated fatty acids or by the descarboxylation of β-ketoacids present in milk fat. Furthermore, numerous authors have reported that high pressure enhances oxidation of free fatty acids, leading to the formation of ketone volatile compounds (Oey *et al.*, 2008; Van Der Plancken *et al.*, 2012; Garrido *et al.*, 2015).

During HPP, there is an increase in temperature due to adiabatic heating. The magnitude of this change depends on the compressibility of the substance and its specific heat (Trujillo, 2002). Vazquez-Landaverde, Torres, and Qian (2006) observed an increase in aldehyde concentration when milk was subjected to HPP under 620 MPa at 60 or 80 °C during 1, 3 or 5 min, and suggested that this might be due to higher solubility of oxygen under high pressure, which could enhance the formation of hydroperoxides, leading to the production of more aldehydes.

Throughout almost all storage period, aldehydes/ketones content of thermally treated and 600/5 samples was statistically higher than that of 600/15 samples ($p<0.05$), except on the 28th day, where all samples presented statistically similar ($p>0.05$) values (**Table 16**). Garrido *et al.* (2015) observed a significant decrease ($p<0.05$) on polyunsaturated fatty acids content (most prone to oxidation) of human milk treated at 600 MPa for 6 min. They suggested that the high content of aldehydes in HPP treated milk, could be due to fracture of fat globules, where lipids were immersed, as a result of pressure intensity.

Aliphatic hydrocarbons were the major VOCs found on cream samples, presenting no regular tendency throughout storage days at all conditions (**Table 16**). Generally, the tendency for all conditions was to increase ($p<0.05$), however, on some storage days, the levels also decreased significantly ($p<0.05$). Their levels on initial raw cream were statistically similar ($p>0.05$) to both thermal and HPP samples, however, between both treatments, their content was statistically different ($p<0.05$), being aliphatic hydrocarbons content higher on thermally treated samples. Accordingly, Chugh *et al.* (2014) observed a significant increase in hydrocarbons compounds after heat treatment of skim milk.

Lastly, lactones, which were detected in very low levels in all cream samples, are reported in literature to be related with lipid degradation, being formed by cyclicization of γ- and δ-hydroxyacids (Juan *et al.*, 2010). HPP samples levels of lactone were statistically similar ($p>0.05$) to that of initial raw cream. Furthermore, their levels on all samples increased significantly ($p<0.05$) throughout all storage time, being always higher on HPP samples than on thermal treated ones (**Table 16**).

Preserving the original VOCs in dairy cream is of important concern, since variations in the profile of these compounds may negatively affect its quality. In summary, initial

treated samples did not vary significantly when compared to initial raw cream. Furthermore, generally all compounds presented a tendency to increase throughout storage period, without major differences between thermally pasteurized and HPP samples.

PART II

CARRIED OUT IN FOGGIA, ITALY

CHAPTER III – RESULTS AND DISCUSSION

THIS SECTION COMPRISES ALL THE OBTAINED RESULTS REGARDING THE EFFECT OF ULTRASOUND TREATMENT ON THE STABILITY AND CHEMICAL CHARACTERISTICS OF EMULSIONS BASED ON EVOO

1. Influence of operating variables of ultrasound treatment on the type and stability of the emulsions

To evaluate the type of emulsion generated by the ultrasound treatment without the addition of emulsifiers, the electrical conductivity (EC) of each sample, produced by changing the power values, amplitude and cycle pulse, was measured. The general equation below demonstrates that only power and amplitude had a significant (p<0.05) impact on the electrical conductivity:

$$EC\ (\mu S) = 1193.17 - 12.57 \times [Power] - 10.90 \times [Amplitude] + 0.19 \times [Power] \times [Amplitude]$$

Moreover, the percentages of power and amplitude, individually, showed a negative linear effect on this parameter and a positive interaction between them. In particular, the iso-response surface showed that the increase of EC, i.e. the formation of an O/W emulsion type, was obtained at the highest or the lowest values of power and amplitude (**Figure 23**). In addition, a tendency to reverse emulsion was observed when setting the lowest percentage of power with increasing amplitude percentages and *vice versa* during the ultrasound treatment.

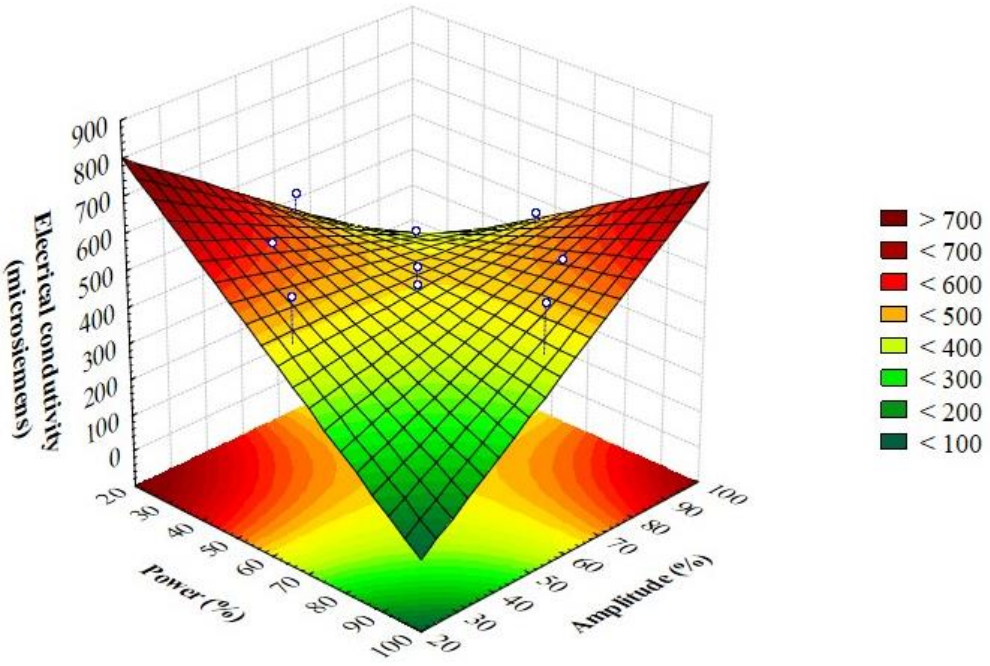


Figure 23 - Iso-response surface of electrical conductivity (μS) of emulsions produced by ultrasound treatment at different percentages of power and amplitude.

To evaluate the effect of ultrasound parameters on the emulsion's oil and water interface, the density values were measured. In fact, if the interface is considered as a layer of finite thickness, materials in the interface will have densities of intermediate values between the density of oil and water. In this case, the density of emulsion resulted equal to water density (1 g/cm^3), for every treatment that was carried out. This is probably due to the low oil phase volume fraction in the emulsion in comparison to the continuous water phase. This behaviour emphasizes that the application of ultrasound treatments on emulsions without the addition of emulsifier, favours the formation of an O/W emulsion.

The general equation (below) related to the percentages of emulsion stability (ES), highlights that also in this case, only the power and amplitude had a significant effect on this parameter.

$$ES\ (\%) = 111.63-0.54\times[\text{Power}]-0.56\times[\text{Amplitude}]+0.01\times[\text{Power}]\times[\text{Amplitude}]$$

Moreover, the iso-response surface showed that the maximum percentage of emulsion stability was obtained when the highest or the lowest values of power and amplitude were applied (**Figure 24**).

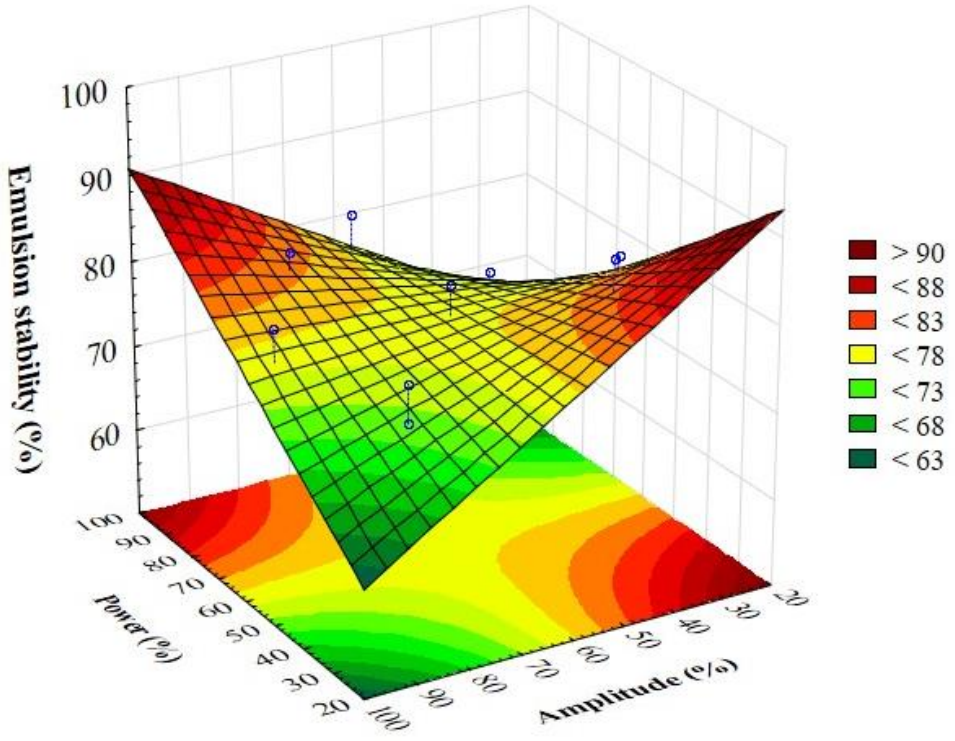


Figure 24 - Iso-response surface of emulsion stability (%) produced by ultrasound treatment at different percentages of power and amplitude.

Furthermore, after centrifugation, it was observed some separation of the oil phase in all samples (**Figure 25**). Consequently, to obtain an emulsion made of EVOO, it was necessary to add an emulsifier, to assist the ultrasound treatment in order to obtain a stable emulsion, that even after a centrifugation treatment does not display a separated oil phase.

Figure 25 – Visual appearance of emulsion produced by ultrasound treatment composed by mineral water and EVOO, without emulsifier.

For this reason, the three emulsifiers were tested in order to choose the type and the amount of emulsifier necessary to obtain a stable emulsion based on EVOO. Moreover, to improve the microbiological stability of the emulsion, lemon juice was added, thus reducing the pH value to less than 4.5.

2. Evaluation of the type and percentage of emulsifier

In order to obtain a stable emulsion in a short time, avoiding excessively heating the samples and preserving important components such as polyphenols, it was necessary to apply high energy by ultrasound treatment. As a function of emulsion's stability, discussed in the previous section, the emulsions prepared with emulsifier were produced using the maximum values of power and amplitude (100%) and medium values of cycle pulse (60%).

Samples prepared with different percentages of EVOO, water, soy lecithin and lemon juice, produced by ultrasound treatment yield unstable emulsions, with a great oil phase separation (**Figure 26**).

Figure 26 - Emulsion made of EVOO, mineral water, soy lecithin, lemon juice after 24 h of storage at room temperature.

Similar results were obtained for emulsions prepared with EVOO, sucrose fatty acid esters (emulsifier), lemon juice and mineral water (**Figure 27**).

Figure 27 – Centrifugated emulsion based on EVOO, sucrose fatty acid esters, lemon juice and mineral water right after ultrasound treatment.

A positive and different behaviour was observed for the emulsion prepared with MDG. In fact, the obtained emulsion was stable, even after centrifugation and storage for 7 days at room temperature (**Figure 28**).

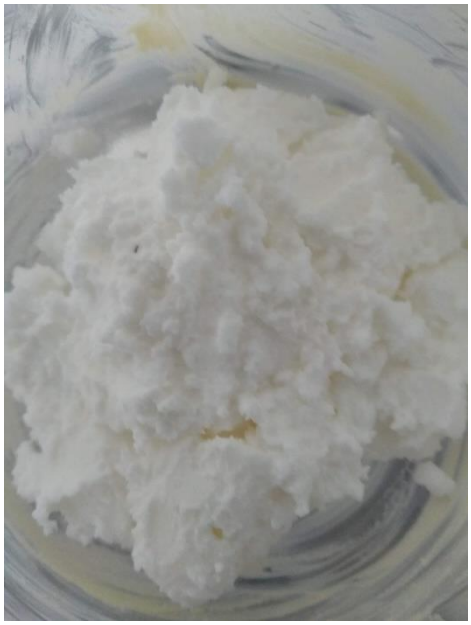


Figure 28 - Stable emulsion based on EVOO, MDG, lemon juice and mineral water submitted to centrifugation and stored at room temperature for 7 days.

Many samples were prepared with different amounts of EVOO and MDG in order to determine the optimal percentage of emulsifier (**Table 8**). All samples presented values of electrical conductivity equal to 0 μS, meaning that the type of emulsion was W/O, due to the fact that electric current does not pass through the medium when oil is the continuous phase (Raviadaran *et al.*, 2019).

3. Solubility test

Results of solubility test showed that emulsions resulted soluble only in hexane, which is an apolar solvent. Moreover, no separation was observed during the dissolution of the emulsion in this solvent, highlighting the fact that the continuous phase was made of lipidic fraction.

4. Evaluation of the dynamic viscosity

The dynamic viscosity was evaluated in order to select the percentages of EVOO and MDG that favoured the formation of a stable emulsion with high resistance to shear stress. The general equation (below) shows that the percentages of EVOO and MDG had a linear positive effect, meaning that the viscosity of emulsion increased with increasing amount

of oil and emulsifier, being the maximum values obtained with the maximum percentages of both parameters (**Figure 29**).

$$\textit{Dynamic viscosity (mPa*s)} = -191.375+0.576\times[\text{MDG}]\times[\text{EVOO}]$$

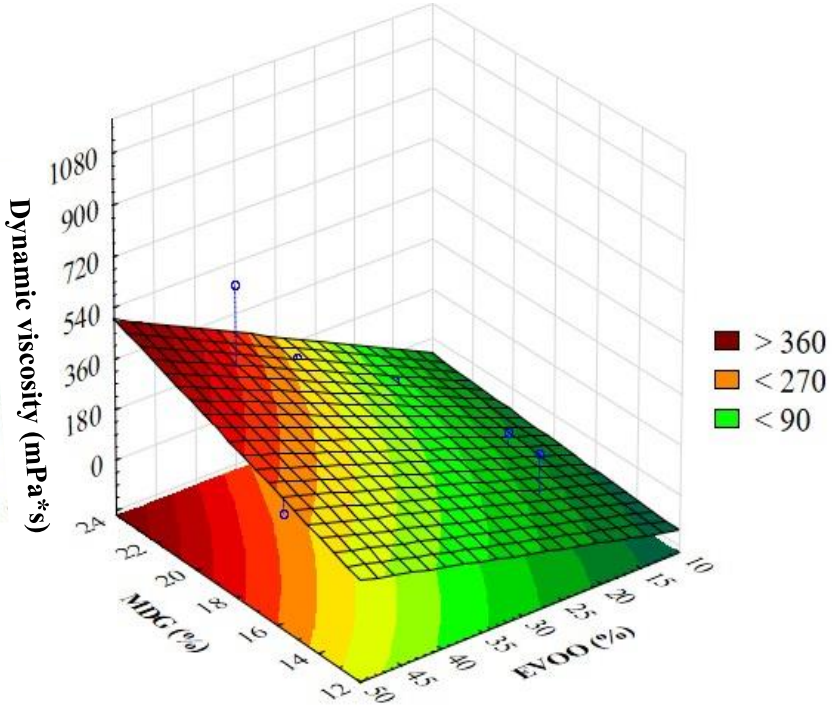


Figure 29 - Iso-response surface of the dynamic viscosity (mPa*s) of emulsions made with different percentages of EVOO and MDG.

5. Chemical composition of each EVOO

In order to characterize the different EVOO used to prepare the emulsions, their polyphenol content and also their technological parameters and chemical compositions were analysed by a NIR spectrometer (**Table 17**).

With high and medium levels of polyphenols content (Del Coco *et al.*, 2014), Coratina and Peranzana, respectively, are traditional cultivars of the province of Foggia, area where this research was carried out. Arbequina and A07 are, respectively, medium-low and low polyphenols content cultivars, both of recent introduction in the Province of Foggia but with rapidly increasing cultivated surfaces.

Table 17 - Chemical composition of the four EVOO cultivars studied determined by Near-infrared spectroscopy (NIR).

Samples	Arbequina	A07	Peranzana	Coratina
Peroxides (meq 0_2/100g)	8.05	7.91	5.90	5.74
Total poliphenols (mg/kg)	451.16	410.76	497.73	658.03
Hydroxytyrosol (mg/kg)	15.56	15.18	14.41	16.45
Tyrosol (mg/kg)	18.12	16.93	12.50	17.85
Palmitic acid (%)	12.83	13.04	12.14	11.93
Palmitoleic acid (%)	0.91	1.00	0.86	0.59
Heptadecanoic acid (%)	0.05	0.05	0.05	0.05
Heptadecenoic acid (%)	0.08	0.09	0.08	0.09
Stearic acid (%)	2.10	2.04	2.22	2.22
Oleic acid (%)	72.26	70.00	71.99	73.33
Linoleic acid (%)	9.89	12.12	10.57	9.68
Linolenic acid (%)	0.71	0.72	0.77	0.75
Arachidic acid (%)	0.39	0.39	0.41	0.43
Eicosanoic acid (%)	0.32	0.32	0.33	0.35
Total tocopherols (mg/kg)	224.14	230.63	176.19	207.23

6. Evaluation of polyphenols content on the emulsions' stability

Four different varieties of EVOO were used to prepare the emulsions in order to evaluate the effect of different polyphenol content on the emulsions' stability prepared by ultrasound treatment. The sunflower seed oil was considered as control for both the absence of polyphenols and the higher content of monounsaturated fatty acids when compared to other seed oils.

The a_w and pH values (**Table 18**) were evaluated in order to verify the necessity of submitting the emulsions to further sanitization treatments, in order to make these food preparations microbiologically stable. All samples presented values of a_w and pH quite similar among them. The medium value of a_w and pH obtained was 0.998 ± 0.001 and 3.61 ± 0.02, respectively. The high value of a_w in all samples highlighted the need of a further stabilization treatment. Nevertheless, the low values of pH indicated that a pasteurization treatment and refrigerated storage were enough to keep the emulsions safe.

Table 18 - aw and pH values of each emulsion. Results are presented as mean ± standard deviation.

	Sunflower	Arbequina	A07	Peranzana	Coratina
pH	3.61 ± 0.01	3.60 ± 0.01	3.59 ± 0.01	3.64 ± 0.01	3.60 ± 0.01
aW	0.999 ± 0.01	0.998 ± 0.01	0.996 ± 0.01	1.00 ± 0.01	0.997 ± 0.01

Figure 30 shows the creaming index of the emulsions made with the vegetable oils with different content in total polyphenols. It was observed that the use of EVOO with

more than 451 mg/kg total polyphenol content contributed to an increase (p<0.05) in emulsion's stability, which is represented by the low creaming index percentages (Karami *et al.*, 2019). These data are in agreement with those obtained by Di Mattia *et al.* (2015). Emulsions based on EVOO containing the lowest total polyphenol content (A07) presented some separation of the oil phase, thus not yielding a stable emulsion. Therefore, the emulsion based on this oil will be no longer analysed on the next sections (***"section 6 – Total polyphenols content of emulsions"*** and ***"section 7 – Microbial analysis"***).

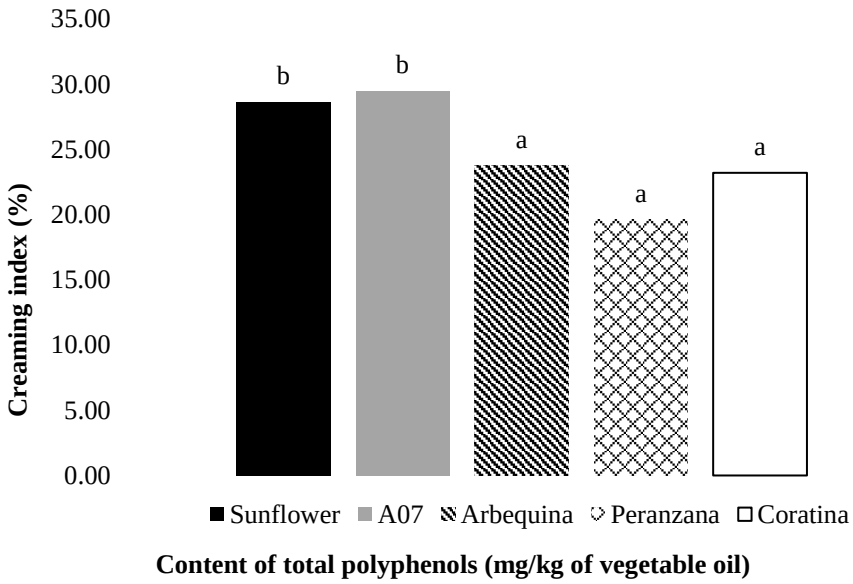


Figure 30 - Creaming index of emulsions made with vegetable oil with different total polyphenol content. Different letters denote significant differences (p<0.05) between each emulsion (a-b).

The dynamic viscosity of emulsions submitted and not submitted to centrifugation was determined in order to evaluate the effect of a destabilization treatment on the rheological properties of emulsions based on sunflower oil, thus evaluating the resistance of emulsions independently of the polyphenol content. **Figure 31** shows that both samples presented a pseudo plastic behaviour, i.e., the increase of shear rate involved a decrease of dynamic viscosity. Moreover, the centrifugated samples showed the highest values for the minimum shear rate. This is probably due to the effect of compacting the emulsion structure and removing the non-emulsified water during centrifugation. Also, both samples showed an increase of dynamic viscosity after the third cycle of compression, which could be due to the formation of adhesive forces between the lipid continuous phase.

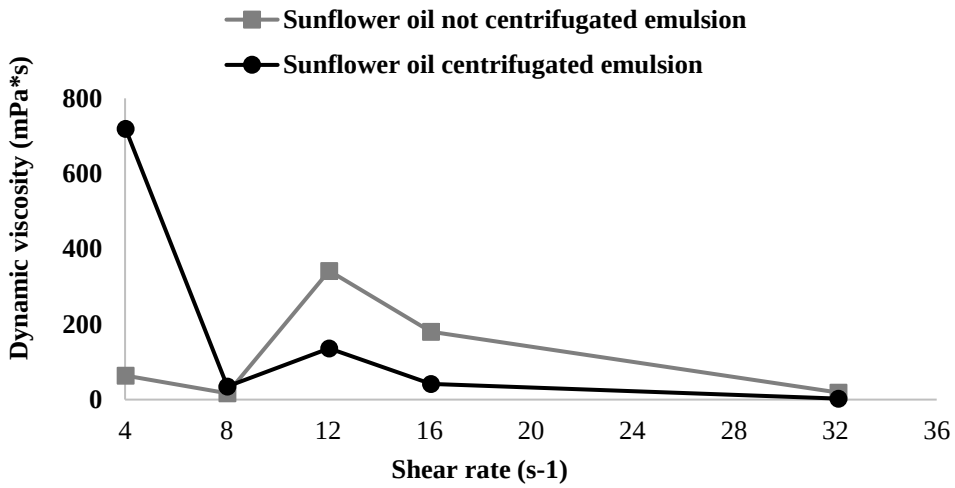


Figure 31 - Dynamic viscosity values as a function of the shear rate of emulsions made of sunflower seed oil, submitted and not to centrifugation treatment.

As a function of these results, and to evaluate the effects of different types of vegetable oils on emulsion's rheological properties without stressing their structure by centrifugation, the dynamic viscosity was measured immediately after ultrasound treatment.

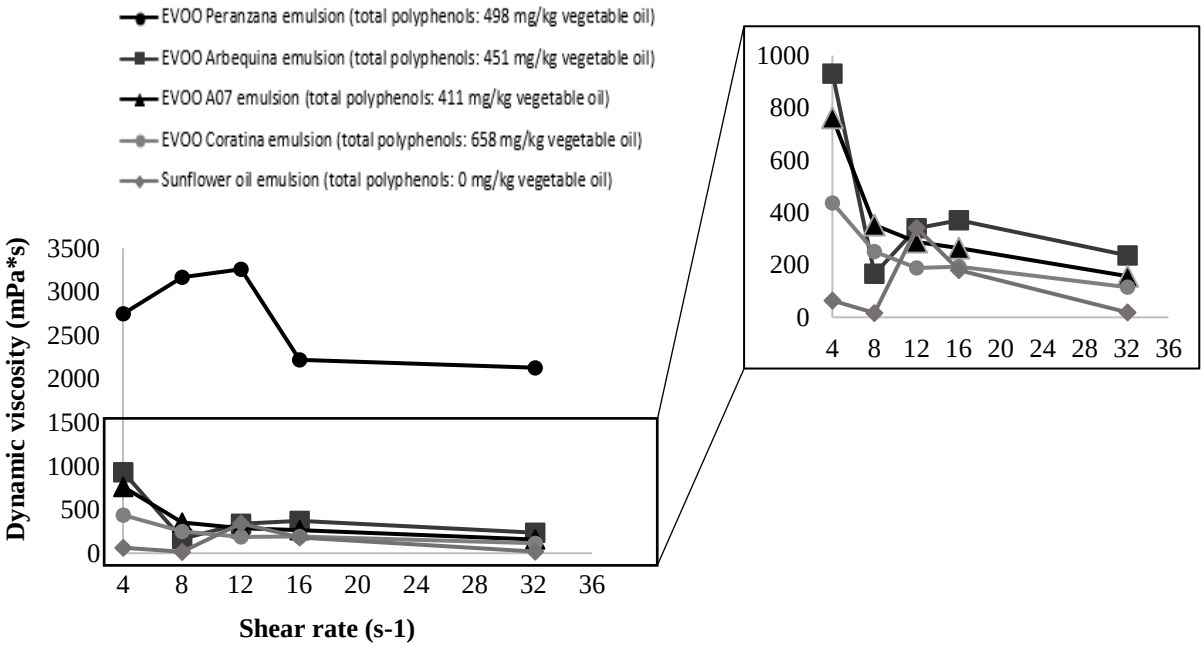


Figure 32 - Dynamic viscosity values as a function of shear rate of emulsions produced with different vegetable oils, with different total polyphenol content.

Results showed that all samples, except for emulsion made with Peranzana EVOO, presented a pseudo plastic behaviour (**Figure 32**). On the contrary, emulsions made with this oil showed not only a high value of dynamic viscosity at low shear rates but also an increase of viscosity with increasing shear rate, even if a decrease was observed after the

fourth cycle. This behaviour is typical of viscous-plastic fluids. This type of non-Newtonian fluid behaviour is characterized by the existence of a threshold stress (called yield stress or apparent yield stress), which must be exceeded for the fluid to deform (shear) or flow (Balmforth and Frigaard, 2007). Conversely, such a substance will behave like an elastic solid (or flow in masse like a rigid body) when the externally applied stress is less than the yield stress. Of course, once the magnitude of the external yield stress exceeds the value of yield stress, the fluid may exhibit Newtonian behaviour (constant value of viscosity) or shear-thinning characteristics.

It is possible to hypothesize that the different behaviour of the emulsion produced by Peranzana EVOO is due to the fine and homogeneous dispersion of water droplets inside the lipid continuous phase. The water droplets are, probably, trapped inside of fat crystals formed by the emulsifier solidification. In fact, MDG is constituted by 90% of saturated fatty acids that solidify at room temperature. In order to verify this hypothesis, some optical microscope images were acquired (**Figure 33**). It was possible to observe that the images did not show a clear dispersion of a component inside the continuous phase, but a heterogeneous mass. This could be due to the little dimension of the water droplets dispersed inside the lipid phase.

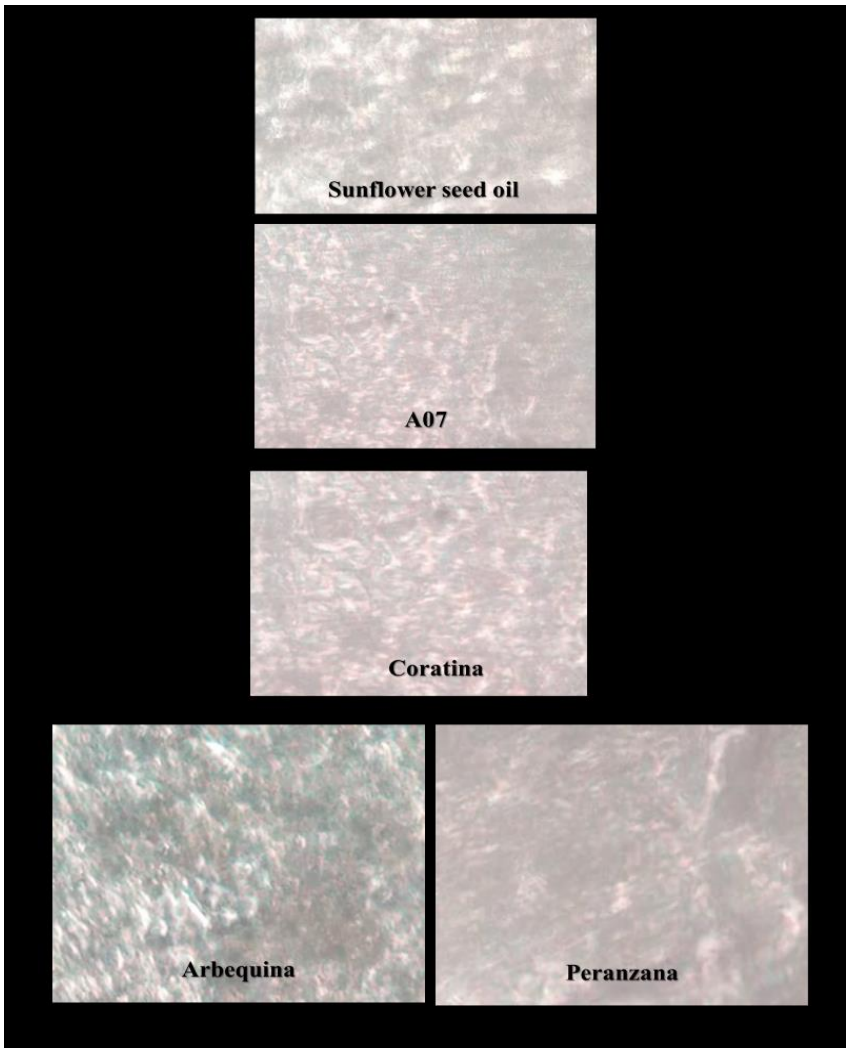
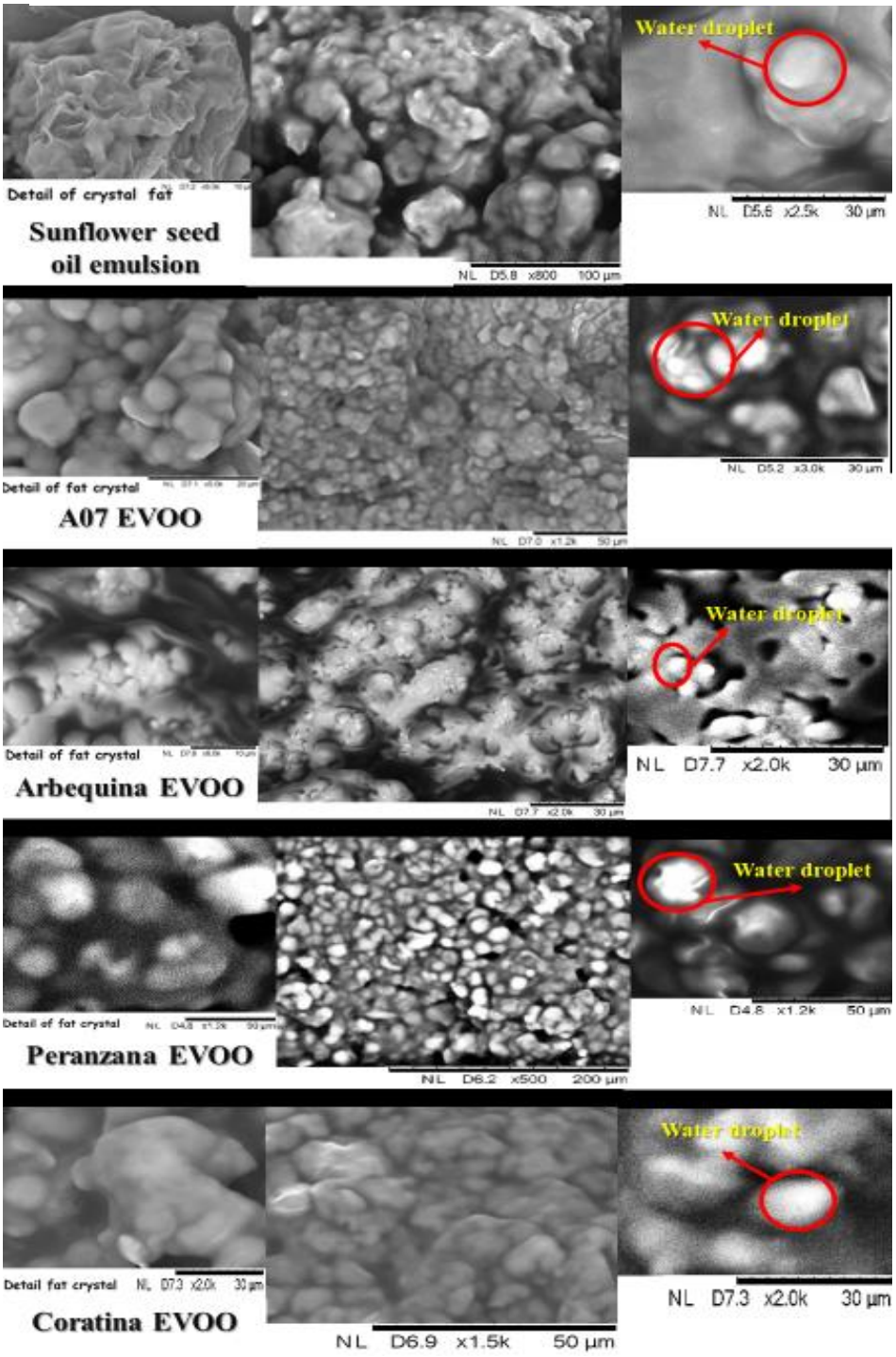


Figure 33 - Optical microscope images at 40x magnification of emulsions made with different vegetable oils.

Figure 34 shows the SEM images of emulsions made with different vegetable oils. It was observed that each emulsion presented a different shape of fat crystals, which could be due to the influence of the different chemical composition of oils on the speed of lipid crystallization. Moreover, emulsions made with Peranzana EVOO were the only samples that presented a homogeneous distribution of water droplets, highlighting a clear dispersion of water inside the lipid phase. Emulsions based on Coratina EVOO (higher polyphenol content), presented a more open and coarse structure, which is in accordance with Di Mattia *et al.* (2015) who observed this type of structure in EVOO with increasing polyphenols content.

Figure 34 - SEM images of emulsions made with different vegetable oils.



7. Total polyphenols content of emulsions

As mentioned before, phenolic compounds are an important class of metabolites that play an important role in human health and development, and are also normally related to oil's shelf-life, and to its sensory aspects and biological properties. The total content of phenolic compounds was investigated in order to understand their behaviour after

submitting each emulsion to HPP. In **Table 19** it is possible to compare the total phenolic content in equivalents of gallic acid on raw (initial) emulsion and after HPP treatment. The total polyphenol content of each emulsion is in agreement with the values determined for each EVOO, by NIR, being the emulsion based on Coratina with the highest values, followed by Peranzana and then Arbequina.

Pressure treatment produced a slight but non-significant (p>0.05) increase on total phenolic content of each EVOO emulsion, except for Coratina, where the initial content of phenols was significantly higher (p<0.05) than after HPP. Given these results, it appears that phenols are relatively resistant to pressure processing (Patras *et al.*, 2009).

Table 19 - Total phenolic content of initial and pressure processed EVOO emulsions. Different letters represent statistical differences (p<0.05) between initial (raw) and after HPP emulsions.

Samples	Conditions	Total phenolics (mg gallic acid/g)
Arbequina	**Initial**	0.115 ± 0.01[a]
	500 MPa/5 min	0.136 ± 0.01[a]
Coratina	**Initial**	0.241 ± 0.01[b]
	500 MPa/5 min	0.193 ± 0.01[a]
Peranzana	**Initial**	0.129 ± 0.01[a]
	500 MPa/5 min	0.132 ± 0.01[a]

8. Microbial analyses

In this study, the total aerobic mesophiles and yeasts and moulds microbial counts were determined through all the conditions tested.

8.1. Total Aerobic Mesophiles analyses

Total aerobic mesophiles on each emulsion were quantified before (initial), immediately after HPP (day 0) and after 20 days of storage (room temperature). The initial load (0D) of TAM on emulsions based on Arbequina (Arbeq. raw), Coratina (Corat. raw) and Peranzana (Peranz. raw) EVOOs was 3.69 ± 0.10, 4.04 ± 0.01 and 2.09 ± 0.09 log CFU/g, respectively (**Figure 35**). Similarly, Koidis, Triantafillou and Boskou (2008) analysed an olive oil for TAM and YM and reported that their initial counts ranged from below the detection limit to 3 log CFU/mL.

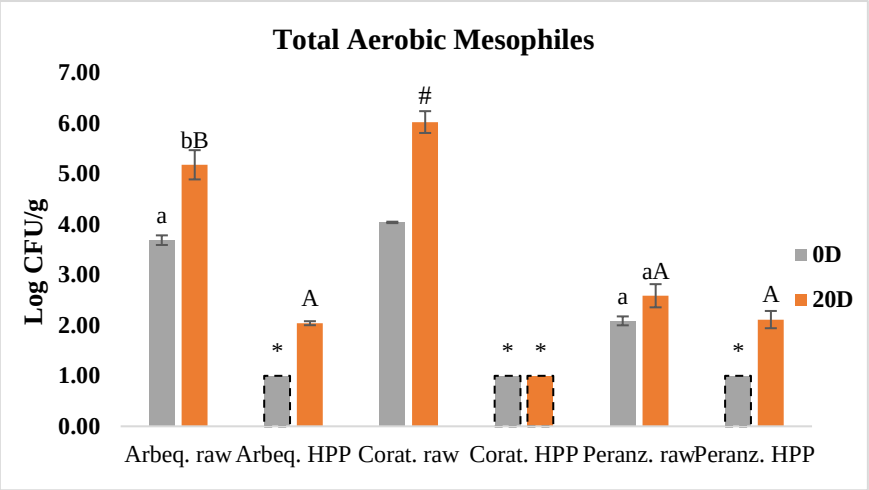


Figure 35 - Microbial growth of TAM on raw emulsion and after pressure treatment under 500 MPa for 5 min. Analyses were made on raw emulsion, right after processing (0D) and after 20 days of storage (20D) of both raw and processed emulsions. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Upper/lower case letters denote significant differences (p<0.05) between raw and treated emulsions at the same storage day (A-B) and between different storage days of each condition (a-b), respectively.

As represented in **Figure 35**, after HPP, initial TAM counts of all samples were successfully decreased to values below the detection limit (≤ 1.00 log CFU/mL).

Regarding emulsions based on Arbequina EVOO, throughout storage at room temperature, it was observed a significant increase (p<0.05) of initial TAM counts on the non-treated samples. During storage, TAM counts of processed samples increased from below detection limit to 2.04 ± 0.04 log CFU/g, being still significantly lower than raw samples at the 20th day.

Emulsions based on Coratina EVOO presented the highest values of TAM counts on raw samples, increasing to values above 6.00 log CFU/g after 20 days of storage. However, HPP samples, both initially and after 20 days of storage, presented TAM counts below the detection limit. Anton *et al.* (2001) found that HPP at 200 and 500 MPa applied on acidic (pH=3.0) and neutral (pH=7.0) emulsion, respectively, can effectively decrease the total bacteria count (> 4.0 $\log_{10}$ cycle).

Lastly, emulsions based on Peranzana EVOO presented similar (p>0.05) TAM counts between initial raw samples and after 20 days. HPP samples (0D) presented TAM counts below detection limit, however throughout storage, it was observed an increase to 2.11 ± 0.17 log CFU/g.

8.2. Yeasts and moulds analyses

Yeasts and moulds (YM) can be responsible for various degrees of food decomposition, being able to proliferate in a wide range of pH (2-9), temperature (5-35 °C), and water activity (a_w>0.85), with some species capable of growth above or below these ranges, which allows them to also colonize and grow in a wide range of foods. Furthermore, many fungal species are known to produce mycotoxins that may pose a greater concern for public health (Kalinowski *et al.*, 2003; Tournas *et al.*, 2006).

YM on each emulsion were quantified before (initial), immediately after HPP (day 0) and after 20 days of storage (room temperature). The initial load of YM on emulsions made with Arbequina, Coratina and Peranzana EVOOs was below the detection limit, 4.25 ± 0.02 and 2.66 ± 0.18 log CFU/g, respectively (**Figure 36**).

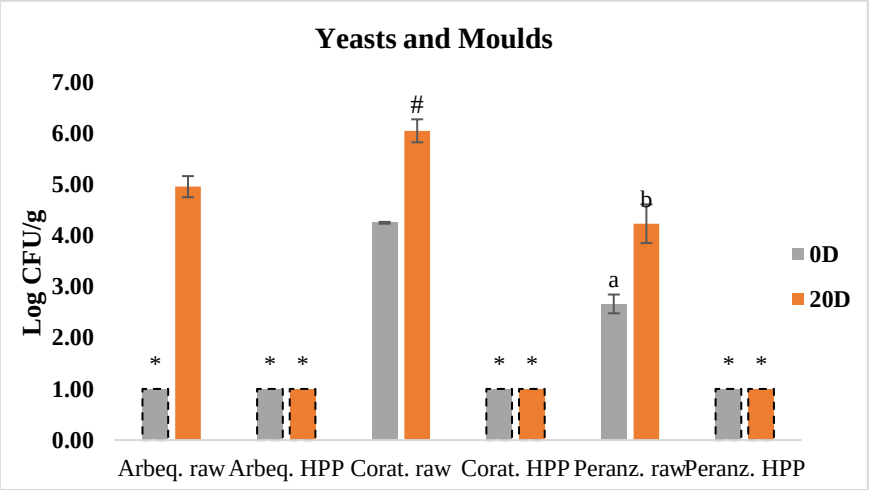


Figure 36 - Microbial growth of YM on raw emulsion and after pressure treatment under 500 MPa for 5 min. Analyses were made on raw emulsion, right after processing (0D) and after 20 days of storage (20D) of both raw and processed emulsions. Bars with * and # represent microbial loads below the detection limit (lower than 1.00 log CFU/mL) and above 6.00 log CFU/mL, respectively. Upper/lower case letters denote significant differences (p<0.05) between raw and treated emulsions at the same storage day (A-B) and between different storage days of each condition (a-b), respectively.

Yeasts and moulds are the microorganisms most sensitive to HPP (Martínez-Rodríguez *et al.*, 2012), thus in all HPP samples (**Figure 37**), YM were not able to recover after the application of 500 MPa, remaining below the detection limit (≤ 1.00 log CFU/mL) throughout the storage period.

Non-treated emulsions based on Arbequina EVOO had its YM increased from counts below the detection limit to 4.95 ± 0.21 log CFU/g throughout storage. On Koidis *et al.* (2008) study, the authors observed that the mould population on virgin olive oil were

initially lower than 1 log CFU/g, but increased continuously during storage for 3 months at 22 ± 1 °C.

Similarly to TAM counts, initial YM counts of emulsions based on Coratina EVOO increased to above 6.00 log CFU/g after 20 days of storage. As for HPP samples, they remained below detection limit even after 20 days of storage at room temperature. Anton *et al.* (2001) reported that acidic and neutral emulsions treated at 200 and 500 MPa, respectively, were able to reduce the total population of yeasts and moulds up to 1.0 – 2.0 $\log_{10}$ cycles.

As for emulsions based on Peranzana EVOO, initial YM counts significantly increased after 20 days of storage.

8.3. General analysis

This is the first time that EVOO based emulsions with different total phenolic content are submitted to HPP and their behaviour is studied throughout a storage period at room temperature.

The difference on the microbiological counts of each emulsion based on different EVOO varieties can be attributed to their different total phenolic content, which can interfere with the viability of each microorganism. The antibacterial effect of phenolic compounds is highly correlated with their concentration as well as their profile on each type of emulsion (Zullo *et al.*, 2018).

Overall, samples that underwent 500 MPa for 5 min kept both TAM and YM below the detection limit throughout all storage at room temperature. This means that after HPP, it was not necessary to store samples at refrigeration temperature, since room temperature was enough to keep emulsions stable, at least for 20 days. These results highlight the efficiency of HPP to inhibit or slow microbial growth over time.

CHAPTER IV – CONCLUSIONS

THIS SECTION COMPRISES THE MAIN FINDINGS OBTAINED THROUGHOUT THIS THESIS, REGARDING BOTH THE WORK DONE IN AVEIRO AND FOGGIA

This is one of the first works that studied the overall effect of HPP on microbial and physicochemical properties of raw dairy cream, as an alternative to standard thermal pasteurization. Concerning cream's microbial load, by the 51st day of storage, thermally pasteurized samples presented microbial counts above 6.00 log CFU/mL, while HPP samples were still microbiologically acceptable. As for the effect of HPP on inoculated microorganisms, it was possible to conclude that, although HPP at 600/15 was able to reduce microbial load to lower counts then 600/5, similar results were observed for both treatments over the storage period, indicating that inactivation effect is little dependent on process time.

In general, regarding physicochemical analysis, pH, colour, viscosity and FA were not significantly altered (p>0.05) by the different processing conditions and storage, while viscosity presented higher values on HPP samples. Furthermore, VOCs of all treated samples presented a tendency to increase throughout storage period, particularly acids and aliphatic hydrocarbons.

According to what is described in the literature, pasteurized cream's expected shelf-life is < 3 weeks (Deosarkar *et al*., 2016), therefore, the outcomes of this work are promising, once HPP was able to microbiologically extend cream's shelf-life in at least 30 days. Furthermore, this work also opens the possibility for further studies, namely sensorial analysis, and also its effect on different cream products, such as whipping cream.

Concerning the second part of this thesis, before this work, carried out in Italy, no investigation has been carried out to study the effect of polyphenols on the structure of emulsions produced by ultrasound technology, probably due to the complexity of their formulation, which could hinder the understanding of the role of relatively low concentrations of surface-active bioactive compounds.

It was observed that in order to obtain a stable emulsion by ultrasound it was necessary to use an emulsifier, being MDG the only one that produced a stable emulsion without separation, except for samples prepared with EVOO named "A07". Furthermore, EVOOs with high polyphenol content yield more stable emulsions, as highlighted by the decrease in the creaming index. "Peranzana" EVOO produced an emulsion with homogeneous appearance and good rheological properties. Nevertheless, the polyphenol content did not seem to have an effect on emulsion's rheological properties, since almost all samples presented a similar behaviour.

The phenolic compounds of each emulsion were determined, and generally, no significant differences were observed between raw and HPP emulsions, meaning that phenols can be relatively resistant to the effect of processing.

Finally, microbiological analyses for total aerobic mesophiles and yeasts and moulds were conducted on each emulsion, before, immediately after HPP and throughout storage time. It was observed that HPP was able to inhibit or slow microbial growth over time, keeping the emulsions stable even after storage at room temperature.

Further experiments regarding the effect of HPP on emulsions based on EVOO or other ingredients are of great importance. Under adequate conditions, HPP can modify the functionality of proteins and polysaccharides, changing their emulsifying activity and solubility rates, which can affect not only the preparation, processing and storage of the food products, but can also improve the perception of quality during consumption. Thus, the effect of this technology should be evaluated regarding emulsion's sensorial properties and functionality.

Furthermore, since HPP can affect the structure of food constituents, these pressure-induced changes offer the possibility to develop innovative products, with unique characteristics.

CHAPTER V – REFERENCES

THIS SECTION COMPRISES THE LIST OF THE CONSULTED LITERATURE IN THE SCOPE OF THE PRESENT WORK

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CHAPTER VI – APPENDICES

THIS SECTION COMPRISES ALL THE COMPLEMENTARY INFORMATION MENTIONED ALONG THE VARIOUS THESIS CHAPTERS

APPENDIX A – Cream aroma profile analysis

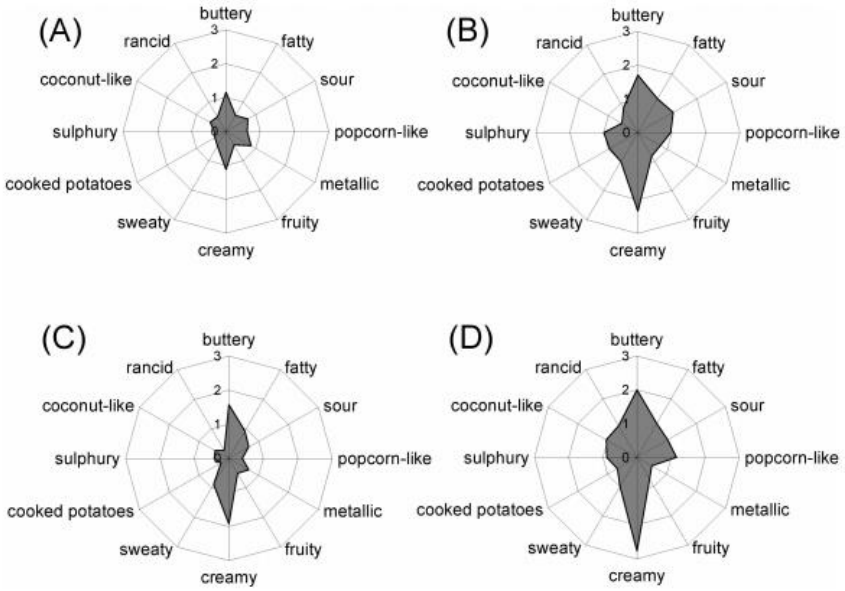

Figure A 1 - Aroma profile analysis of (A) nonheated cream (NHC), (B) high-heat-treated cream (HHC), (C) whipped nonheated cream (WNHC), and (D) whipped high-heat-treated cream (WHHC). Adapted from Schlutt et al. (2007).

APPENDIX B – High pressure processing: commercial application and effect on microorganisms

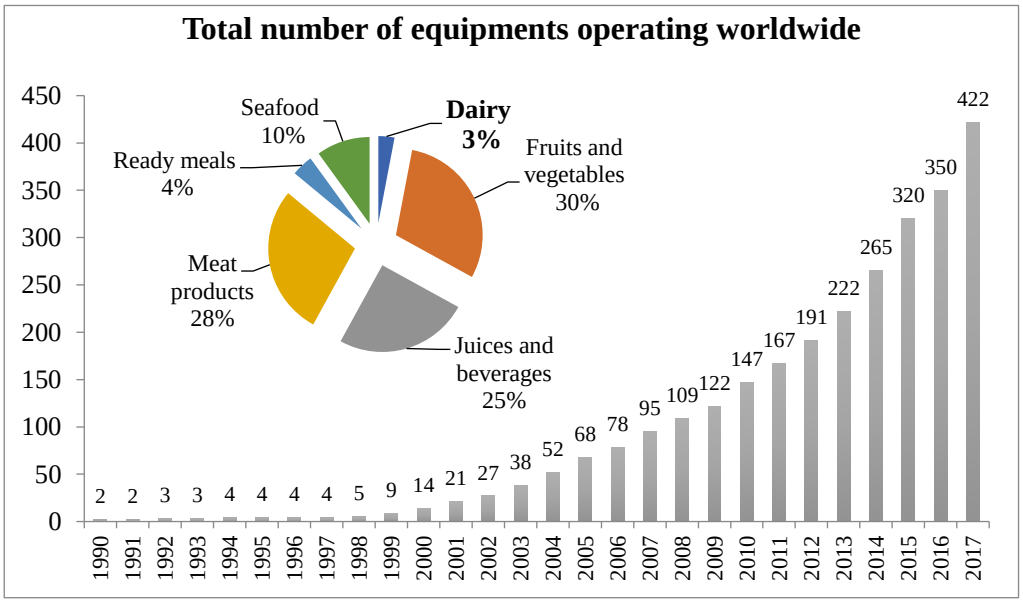

Figure B 1 - Total number of HPP industrial machine currently operating on food industry and categories of foods processed by HPP and its associated percentages. Courtesy of Hiperbaric.

Table B 1 - High pressure treated products on the international market. Adapted from Voigt et al. (2015).

Product group	Product	Company	Country
Dairy products	COL + Colostrum	New Image Natural Health Limited	NZ
	Cheese sandwich filling	Rodilla	
	Soya products	Toby's	
Meat products	Cured smoked ham	Abraham	Germany
	Slice cooked ham	Espuna Co.	Spain
	Sliced ham and cured meats	Santa Maria Foods	Canada
	Chicken sausages	Lou Famous Purdue	USA
	Preservative-free chicken strips	Farms	USA
	Processed poultry	Purdue Farms	USA
	Sliced and diced poultry products	Tyson Foods	USA
	Ready-to-eat sliced meats	Hormel, Kraft	USA
	Sliced, cured and marinated meat	Campofrio	Spain
Seafood products	Oysters	Motivatit Seafood, Goose Point Oysters, Joey Oysters	USA
	Lobsters	Clearwater, Ocean Choice, Seafood 2000	Canada
	Crab	Philips Seafood	USA
	Desalted Cod	Ghezzi	Italy
Vegetables and fruits	Guacamole	Avomex, Freshurized Foods	USA
	Tomato sauces	SimplyFresco	USA
	Avocado products	San Lorenzo	Mexico
		Calavo	USA
	Humus	Hannah International	USA
Juices and beverages	Juice	Pernod Ricard, Pampryl, Ultifruit	France
		Hormel Foods	USA
		Beskyd Frycovice	Czech Republic
		Danny Boy	Australia
		Leahy Orchards	Canada
		Frubaca	Portugal;
		Ata	Italy

Table B 2 - Viability loss of Gram-positive strains after combined treatment of HPP, time and temperature in cryovials containing cell suspensions. Adapted from Alpas et al. (2000).

Bacterial species	Temperature °C	Log10 CFU/mL reduction following pressurization at								
		207 MPa			276 MPa			345 MPa		
		Control	5 min	10 min	Control	5 min	10 min	Control	5 min	10 min
Staphylococcus aureus 485	25	(8.00)	0.40	0.52	(8.00)	0.60	0.70	(8.03)	0.92	1.08
	35		0.50	0.64		0.80	1.10		2.03	2.43
	45		0.60	0.74		3.70	3.89		3.73	3.99
	50		1.77	2.05		5.22	5.82		5.33	6.03
Staphylococcus aureus 765	25	(8.03)	0.59	0.65	(8.03)	0.80	0.95	(8.04)	1.96	2.09
	35		0.63	0.77		1.33	1.89		3.56	4.00
	45		0.71	1.08		3.83	4.73		5.20	5.64
	50		2.65	3.08		8.03	8.03		8.04	8.04
Listeria monocytogenes CA	25	(8.17)	0.47	0.69	(8.12)	0.64	0.78	(8.00)	0.86	2.40
	35		0.79	1.06		1.64	1.84		4.72	5.00
	45		1.03	1.33		2.84	3.28		5.70	6.10
	50		3.03	3.13		6.52	6.82		8.00	8.00
Listeria monocytogenes OH₂	25	(8.09)	0.63	0.79	(8.08)	0.72	0.85	(8.00)	2.64	3.05
	35		1.01	1.14		1.78	1.90		5.05	5.40
	45		1.25	1.49		3.18	3.60		8.00	8.00
	50		3.14	3.25		8.08	8.08		8.00	8.00

Table B 3 - Viability loss of Gram-negative strains after combined treatment of HPP, time and temperature in cryovials containing cell suspensions. Adapted from Alpas et al. (2000).

Bacterial species	Temperature °C	Log10 CFU/mL reduction following pressurization at								
		207 MPa			276 MPa			345 MPa		
		Control	5 min	10 min	Control	5 min	10 min	Control	5 min	10 min
Escherichia coli O157:H7 933	25	(8.18)	0.58	0.70	(8.17)	1.39	1.47	(8.22)	2.52	2.74
	35		0.78	0.88		1.87	2.39		8.22	8.22
	45		2.48	3.28		5.69	6.39		8.22	8.22
	50		3.86	4.00		5.85	6.47		8.22	8.22
Escherichia coli O157:H7 931	25	(8.19)	0.79	0.85	(8.16)	1.46	1.56	(8.14)	3.66	4.00
	35		0.89	0.99		3.38	3.96		8.14	8.14
	45		2.71	3.71		5.88	6.56		8.14	8.14
	50		3.99	4.15		8.16	8.16		8.14	8.14
Salmonella enteritidis FDA	25	(8.24)	0.98	1.04	(8.19)	2.24	2.41	(8.22)	4.12	4.92
	35		1.84	1.98		4.41	5.41		8.22	8.22
	45		3.24	3.40		5.71	6.05		8.22	8.22
	50		5.20	5.46		8.19	8.19		8.22	8.22
Salmonella typhimurium	25	(8.74)	1.54	1.60	(8.64)	3.56	3.86	(8.51)	5.01	5.51
	35		2.14	2.31		4.46	5.86		8.51	8.51
	45		4.14	4.30		7.64	8.64		8.51	8.51
	50		5.60	5.70		8.64	8.64		8.51	8.51

APPENDIX C – Mineral water characteristics

Table C 1 - Commercial mineral water chemical characteristics.

Water characteristics	
Fixed residue at 180 °C	22 mg/L
Hardness	0.9 °F
Sodium content	1.5 mg/L
Electrical conductivity at 20 °C	25.4 mS/cm
Carbon dioxide	2.0 mg/L
pH	6.9
Sodium bicarbonate	10.0 mg/L
Calcium	2.9 mg/L
Fluorides	< 0.10
Nitrates	0.81

APPENDIX D – Ultrasound treatment: device and central composite designs

Figure D 1 - Hielscher ultrasonics - ultrasonic generator UP200St-G (left) and ultrasonic transducer UP200St-T (right).

Table D 1 - Central composite design elaborated to evaluate the effects of ultrasound parameters on stability of emulsion made up of EVOO.

Codified values	Amplitude (%)	Power (%)	Pulse cycle (%)
-1.68	26.36	26.36	13.18
-1	40	40	20
0	60	60	30
1	80	80	40
1.68	93.64	93.64	46.82

Table D 2 - Central composite design elaborated to evaluate the effects of different percentages of emulsifier and EVOO on viscosity of obtained emulsions.

Codified values	Emulsifier in water phase (%)	EVOO (%)
-1.68	25	50
-1	22	40
0	18	30
1	15	20
1.68	12	10

APPENDIX E – Dairy cream rheology

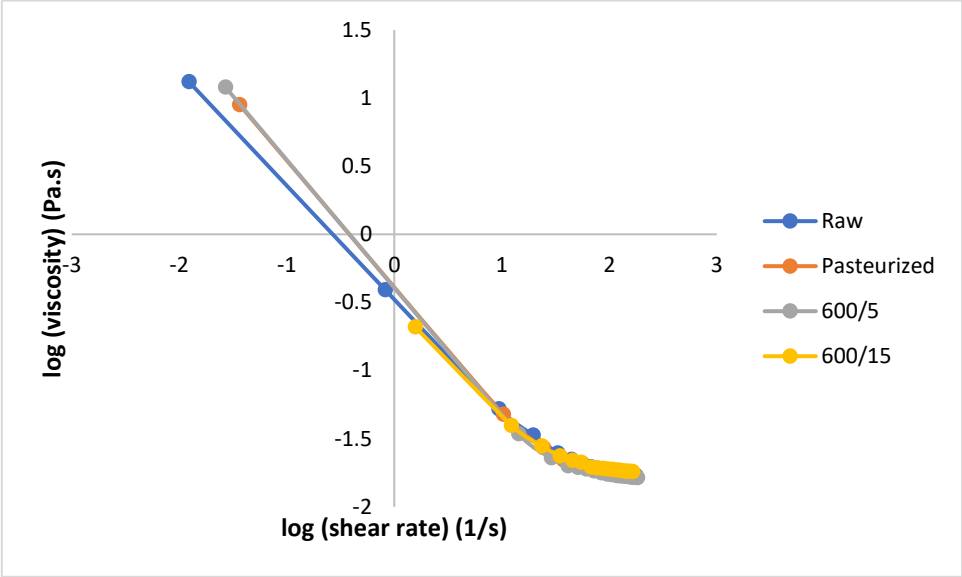

Figure E 1 - Example of initial flow curves of dairy creams before treatment (raw), after thermal pasteurization and after HPP at 600 MPa for 5 and 15 min.